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ENZYME CONTROL OF SPHERE-ROD MORPHOGENESIS IN *ARTHROBACTER CRYSTALLOPOIETES*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

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JOHN ARTHUR FERDINANDUS

Norman, Oklahoma

1969

ENZYME CONTROL OF SPHERE-ROD MORPHOGENESIS

IN ARTHROBACTER CRYSTALLOPOIETES

APPROVED BY

DISSERTATION COMMITTEE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

ENZYME CONTROL OF SPHERE-ROD MORPHOGENESIS IN *ARTHROBACTER CRYSTALLOPOIETES*

CHAPTER I

INTRODUCTION

> The study of biochemical differentiation includes the problem of correlating enzymes and the products of enzyme action with morphological changes during developement. It attempts to explain differentiation in terms of the gradual establishment of specific enzyme patterns and in terms of the sequential accumulation of substrates and products finally found in the characteristic cell types of the mature organism. Biochemical differentiation will be broadly defined as the intrinsic developement of alterations in the enzymatic activities of a cell (58).

Arthrobacter crystallopoietes is a bacterium which undergoes sphere-rod morphogenesis. Its life cycle consists of the germination of coccoidal cells followed by a period of rod-developement. This in turn is followed by cross-septum formation, rod-fragmentation, and eventual production of new coccoidal cells.

The advantage of using *A. crystallopoietes* as a tool for studying cellular differentiation is that *Arthrobacter* has a relatively simple life cycle. In addition, the life cycle is synchronous, can be completed within

30 hrs, and is subject to manipulation by a variety of different compounds. Therefore a study was undertaken to determine, in part, the enzymatic control of sphere-rod morphogenesis.

As a result of determination of changes in enzyme activities of A. crystallopoietes, and their association with changes in intracellular metabolism, a model has been proposed to explain on a molecular level some of the events believed to be occurring during sphere-rod morphogenesis.

CHAPTER II

MATERIALS AND METHODS

Organism. Arthrobacter crystallopoietes ATCC 15481 was grown at 30 C on two types of media, depending upon the type of experiment conducted. For enzyme kinetic measurements, a complete medium was used containing the following per liter: yeast extract, 2.5 g; tryptone, 5 g; glucose, 1 g; agar, 30 g; pH 7.0. For measurements in changes of specific activity of enzymes and metabolites as a function of the life cycle of Arthrobacter, a glucose-minimal salts (GMS) medium plus succinate was employed. The composition of the medium was previously described by Ensign and Wolfe (19).

A 48 hr culture of Arthrobacter was used for the initiation of all experimental work.

Preparation of cell-free extracts. Cells were harvested and washed twice in 0.05 M tris (hydroxymethyl)-amino-methane (Tris)-chloride (pH 7.6). The washed cells were suspended in four times their weight of the same buffer and disrupted by sonic treatment (Blackstone model SS-2C) for 10 min at 4 C.

The crude extract was centrifuged at 30,000 x g for 30 min at 4 C, and the supernatant used for enzymatic assays. Protein was measured by the method of Lowry et al. (34).

Enzyme Assays

All assays were carried out at 25 C, and enzyme activities measured on a Gilford model 2000 recording spectrophotometer. One unit of enzyme activity was defined as an optical density change of 0.001/min. All coupling enzymes employed were checked to be sure they were not contaminated with the enzymes under investigation.

Fructose-1,6-diphosphatase [D-fructose-1,6-diphosphate 1-phosphohydrolase; EC 3.1.3.11]. The method of Rosen et al. (41) was employed. Fructose-6-phosphate produced in the reaction was converted to glucose-6-phosphate by adding excess phosphohexoisomerase. The resulting glucose-6-phosphate was oxidized by adding excess glucose-6-phosphate dehydrogenase and NADP. The reduction of NADP measured spectrophotometrically at 340 nm was proportional to the disappearance of fructose-1,6-diphosphate.

NADP isocitrate dehydrogenase [threo-D-isocitrate: NADP oxidoreductase (decarboxylating); EC 1.1.1.42]. The method of Baldwin and Milligan (4) was used in which the reduction of NADP by isocitrate was followed spectrophotometrically at 340 nm. The reverse reaction, oxidation of NADPH by α-ketoglutarate, was also measured spectrophotometrically at 340 nm.

Malic enzyme [L-malate: NADP oxidoreductase (decarboxylating); EC 1.1.1.40]. Reduction of NADP by malate was measured spectrophotometrically at 340 nm (35).

Lactate dehydrogenase [L-lactate: NAD oxidoreductase; EC 1.1.1.27]. Oxidation of NADH by pyruvate was measured spectrophotometrically at 340 nm (39).

Phosphofructokinase [ATP: D-fructose-6-phosphate 1-phosphotransferase; EC 2.7.1.11]. The method of Ling et al. (33) was employed. To detect production of fructose-1, 6-diphosphate, excess aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, and NADH were added to the reaction mixture. Phosphofructokinase activity was proportional to the rate of oxidation of NADH measured spectrophotometrically at 340 nm.

Glucose-6-phosphate dehydrogenase [D-glucose-6-phosphate: NADP oxidoreductase; EC 1.1.149]. Reduction of NADP by glucose-6-phosphate was measured spectrophotometrically at 340 nm (32).

Phosphoglucomutase [-D-glucose-1, 6-diphosphate: α-D-glucose-1-phosphate phosphotransferase; EC 2.7.5.1]. The procedure of Baldwin and Milligan (4) was followed. Substrate glucose-1-phosphate was converted to glucose-6-phosphate, which was detected by adding excess NADP and glucose-6-phosphate dehydrogenase to the reaction mixture and measuring the rate of reduction of NADP

spectrophotometrically at 340 nm. The rate was proportional to the enzyme activity.

Pyruvate kinase [ATP; pyruvate phosphotransferase; EC 2.7.1.40]. The method of Valentine and Tanaka was used (51). Phosphoenolpyruvate was converted to pyruvate in the presence of excess lactate dehydrogenase and NADH. Oxidation of NADH measured spectrophotometrically at 340 nm was proportional to the activity of pyruvate kinase.

Isocitrate lyase [threo-D-isocitrate glyoxylate-lyase; EC 4.1.3.1]. Activity was measured by the method of Dixon and Kornberg (17) in which glyoxylate produced from isocitrate reacts with phenylhydrazine to form glyoxylic acid phenylhydrazone. The reaction rate was measured spectrophotometrically at 324 nm.

Malate dehydrogenase [L-malate: NAD oxidoreductase; Ec 1.1.37]. Oxidation of NADH by oxalacetate was measured spectrophotometrically at 340nm (23).

Aconitase [Citrate (isocitrate) hydro-lyase; EC 4.2.1.3]. The method of Hanson and Cox (22) was employed whereby substrate isocitrate was converted to cis-aconitate; the reaction was followed spectrophotometrically at 240 nm.

Fumarase [L-malate hydro-lyase; EC 4.2.1.2]. The method of Hanson and Cox (22) was used in which the conversion of malate to fumarate was measured spectrophotometrically at 240 nm.

Phosphohexoisomerase [D-glucose-6-phosphate ketol-isomerase; EC 5.3.19]. The method of Slein (44) was employed whereby fructose-6-phosphate was converted to glucose-6-phosphate in the presence of excess NADP and glucose-6-phosphate dehydrogenase. Reduction of NADP by glucose-6-phosphate, measured spectrophotometrically at 340 nm, was proportional to the activity of phosphohexoisomerase.

Glutamate dehydrogenase [L-glutamate: NADP oxidoreductase (deaminating); EC 1.4.1.4]. The method of Strecker (45) was used in which reduction of NADP by glutamate was measured spectrophotometrically at 340 nm.

PEP carboxykinase [ITP: oxaloacetate carboxy-lyase; EC 4.1.1.32]. The method of Chang and Lane (12) was employed. In the presence of excess pyruvate kinase, lactate dehydrogenase, and NADH, conversion of OAA to PEP was proportional to the oxidation of NADH as measured spectrophotometrically at 340 nm.

Alanine α-ketoglutanate transaminase [L-alanine: 2-oxoglutarate amino transferase; EC 2.6.1.2]. The procedure of Rowsell (42) was used in which alanine was transaminated to pyruvate in the presence of excess lactate dehydrogenase and NADH. The rate of transamination was proportional to the oxidation of NADH as measured spectrophotometrically at 340 nm.

PEP synthase. The method of Berman et al. (8) was employed. Disappearance of pyruvate was measured spectrophotometrically at 324 nm in the presence of excess phenylhydrazine.

Lipase. Activity was measured in the presence of substrate, Ediol, according to the method of Okuda and Fujii (36).

Phosphofructokinase Purification

Phosphofructokinase was isolated from A. crystallopoietes by 10 min sonication in 0.05 M Tris-HCl buffer, pH 8.0, at 4 C. The disrupted cells were centrifuged at 20,000 x g for 30 min at 4 C. All the following procedures were carried out at 4 C. The supernatant was treated with Streptomycin sulphate (0.4 g/ml) to remove nucleic acids and centrifuged at 20,000 x g for 20 min. The pellet was discarded and the supernatant was brought to 50% saturation by addition of solid $(NH_4)_2SO_4$. The precipitated enzyme was removed by centrifugation at 20,000 x g for 20 min and resuspended in 0.05 M Tris-HCl buffer, pH 8.0. The enzyme, purified five-fold, was then dialyzed for 12 hr against 0.05 M Tris-HCl buffer, pH 7.5. In addition, all coupling enzymes were dialyzed for 12 hr against 0.05 M Tris-HCl buffer, pH 7.5, to remove $(NH_4)_2SO_4$.

Metabolite Determination

Free fatty acid determination. Quantitation of free fatty acids from cell-free extracts was done according to the procedure of Okuda and Fujii (36).

Glutamate determination. Glutamate in the free amino acid pool was detected by the method of Hittle (24).

Radioactivity Studies

Permeability of A. crystallopoietes to exogenous glucose. Cells were harvested from a glucose-minimal salts (GMS) medium plus 0.3% succinate at three stages of growth; rod-formation (12 hr), rod-fragmentation (19 hr), and coccoidal growth (30 hr). The cells were washed and suspended in 18 ml of GMS medium containing 1.0 mM glucose. Cellular turbidity was measured at 420 nm in a Spectronic 20 (Bausch & Lomb), and brought to a final optical density of 0.25 in a 18 mm x 150 mm test tube.

The labeled glucose employed had a specific activity of 8.64 mc/mM and was obtained from California Corporation for Biochemical Research (Los Angeles). The cells were pulsed for 10 min at 30 C in 150 ml flask by mixing 18 ml of cellular suspension with 2 ml of D-glucose-6-C-14. The final activity of labeled glucose was 0.1 μc/ml. The cells were shaken at 200 rev/min on a New Brunswick model VS rotary shaker, then filtered through an HAWP (0.45 μm) membrane filter (Millipore Corp., Bedford, Mass.). The membrane filter was washed with cold 0.05 M Tris-HCl buffer,

pH 7.6, dried for 10 min at 80 C, and placed in a vial containing 10 ml of scintillation fluid (0.5% 2,5-diphenyloxazole (PPO) in toluene). Counting was carried out in a Beckman DPM 100 Liquid Scintillation System.

Respiration Studies

Oxygen uptake was measured by standard Warburg techniques described by Umbreit et al. (48). Warburg flasks contained 0.2 ml of 10% KOH (center well), 0.1 ml of 0.1 M glucose, and 2.7 ml of cell suspension. The flasks were equilibrated in air and incubated at 30 C with shaking at 36 excursions/min.

Staining Techniques

Fat material was demonstrated by the staining technique of Conn et al. (14).

Preparation of Lipid Extract

Approximately 4 g (wet weight) of cells of A. crystallopoietes were placed in a Soxhlet extractor for 24 hr. The solvent system employed consisted of 50% ethyl alcohol, 49% acetone, and 1% concentrated HCl. After extraction the solvent was evaporated at 80 C and the crude lipid extract retained.

Kinetic Measurements

Lineweaver-Burk plots were used for the determination of enzyme K_m values and inhibition constants (K_I)

according to the method of Dixon and Webb (18). In addition, plots revealed whether inhibition was competitive, non-competitive, or mixed.

Substrate-binding site interaction coefficients of enzymes were determined from the Hill equation, $\log [v/(V_m-v)] = n \log (S) - \log K_m$. A plot of $\log [v/(V_m-v)]$ as a function of log (S) at various substrate concentrations gave a straight line of slope n. As described by Atkinson et al. (2), the interaction coefficient (n) is a function of the strength of the interaction between substrate binding sites, and a function of the number of substrate binding sites per enzyme molecule. The stronger the interaction, the greater the value of n.

Chemicals

All chemicals employed were obtained through Fischer Scientific Co., Fair Lawn, New Jersey; Sigma Chemical Co., St. Louis, Mo.; or Calbiochem, Los Angeles, California.

CHAPTER III

RESULTS AND DISCUSSION

I. Specific Activities of Enzymes

During rod-formation of A. crystallopoietes there was an observed increase in stainable fat material, and at a time just prior to rod-fragmentation, there was a disappearance of stainable fat material. Similar results had previously been found in Nocardia corallina during its sphere-rod morphogenesis (13). This suggested that fat* metabolism may play an important role in the morphogenesis of both organisms. The problem was attacked in A. crystallopoietes by examining enzymes associated with such pathways as the glycolytic, glyoxylate, pentose cycle, and Krebs cycle. The stages of the life cycle examined were, Stage I, germination of coccoidal cells (7 hr); Stage II, rod-formation (12 hr); Stage III, rod-fragmentation (19 hr); Stage IV, coccoidal growth (30 hr).

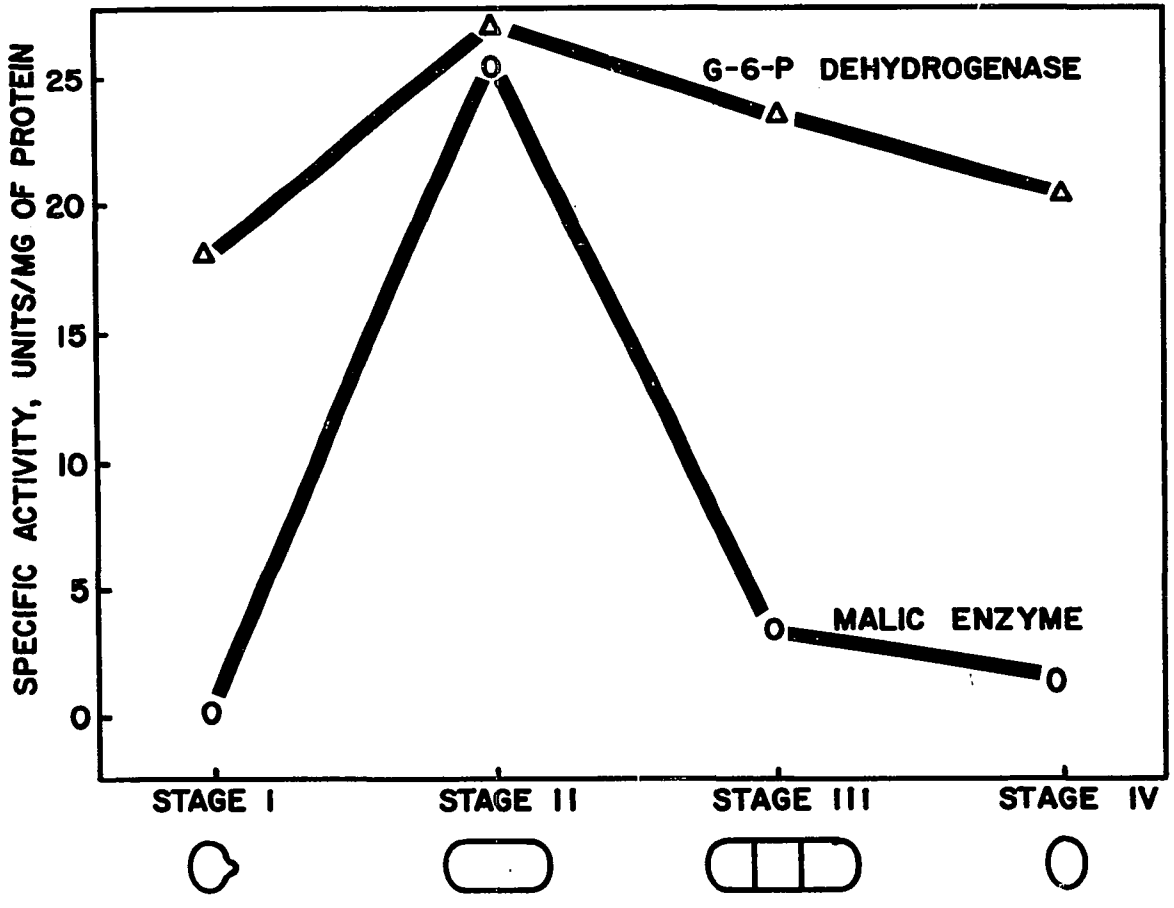
It was found that during rod-formation of A. crystallopoietes both the malic enzyme and glucose-6-phosphate

*Fat is defined as any ether soluble material which upon hydrolysis yields free fatty acids.

dehydrogenase reached their highest intracellular level (Figure 1), coincidental with the time of an observed increase in stainable fat material. Both enzymes produce NADPH and had been shown to have a marked correlation with increased lipogenesis (26, 37, 47, 57, 61). In many cases it had also been found that the amount of NADPH produced from glucose-6-phosphate dehydrogenase was insufficient to account for complete fat synthesis, and that the required amount of NADPH could be supplied by the presence of the malic enzyme (5, 61). When A. crystallopoietes was grown on a glucose-minimal salts medium without succinate, there was no rod-formation, only coccoidal growth. Under these conditions the malic enzyme was completely absent, while the specific activity of glucose-6-phosphate remained essentially constant. Thus it is suggested that rod-formation of A. crystallopoietes is closely associated with increased lipogenesis.

This idea is supported by the fact that when A. crystallopoietes was grown on such reduced compounds as succinate, fumarate, or lactate, all of which can be converted to malate, substrate for the malic enzyme, rod-formation was induced; yet when A. crystallopoietes was grown on more oxidized compounds such as oxalacetate (OAA) or pyruvate, rod-formation was inhibited. Therefore it is believed that the more oxidized compounds, lacking sufficient reducing power, were unable to produce the required

Fig.1.--Specific activities of the malic enzyme and glucose-6-phosphate dehydrogenase as a function of the growth cycle of Arthrobacter crystallopoietes.


SPECIFIC ACTIVITY, UNITS/MG OF PROTEIN
25
20
15
10
5
0
G-6-P DEHYDROGENASE
MALIC ENZYME
STAGE I
STAGE II
STAGE III
STAGE IV

amount of malate needed for lipid synthesis, and thus were unable to stimulate either rod-formation or lipogenesis.

During the morphogenesis of many microorganisms, there appears to be a dependence at a particular stage of growth upon the utilization of an endogenous reserve material as an energy source (58). From measurements of the endogenous oxygen uptake as a function of the life cycle of A. crystallopoietes, it was found that during rod-fragmentation endogenous respiration reached its highest level (Figure 2). This was also coincidental with the time of an observed disappearance of stainable fat material.

Upon examination of the specific activity of several more enzymes as a function of the life cycle of A. crystallopoietes, it was found that lipase reached its highest intracellular level during rod-fragmentation (Figure 3). Thus from respiration and lipase specific activity data, it is suggested that during rod-fragmentation fats are degraded and utilized as an internal energy source.

The gluconeogenic enzyme, fructose-1,6-diphosphatase, was also found to reach its highest intracellular level during rod-fragmentation (Figure 3). Furthermore it was demonstrated by means of pulsing experiments with radioactive glucose (Table 1), that A. crystallopoietes was relatively impermeable to exogenous glucose during both rod-formation (Stage II) and rod-fragmentation (Stage III). Similar results were obtained by the permeability studies

Fig. 2.--Changes in endogenous respiration during the growth cycle of *Arthrobacter crystallopoietes*.

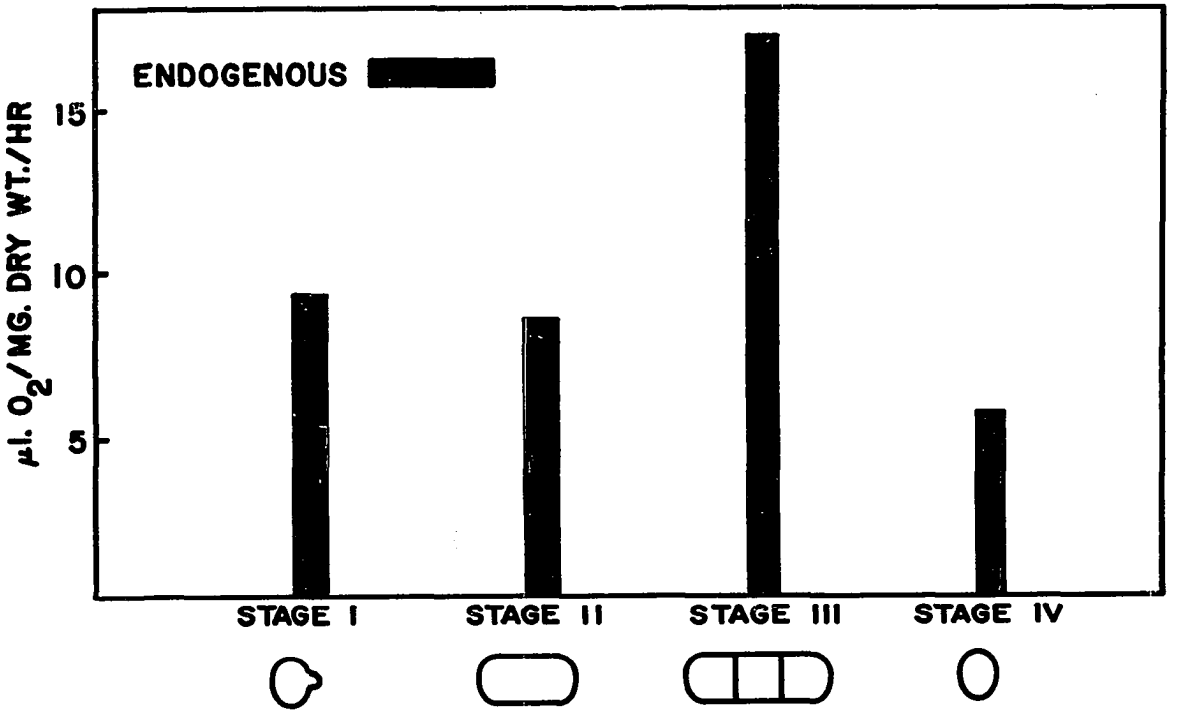

ENDOGENOUS
μl. O2/MG. DRY WT./HR
15
10
5
STAGE I
STAGE II
STAGE III
STAGE IV

Fig. 3.--Per cent change in specific activity of enzymes as a function of the growth cycle of Arthrobacter crystallopoietes.

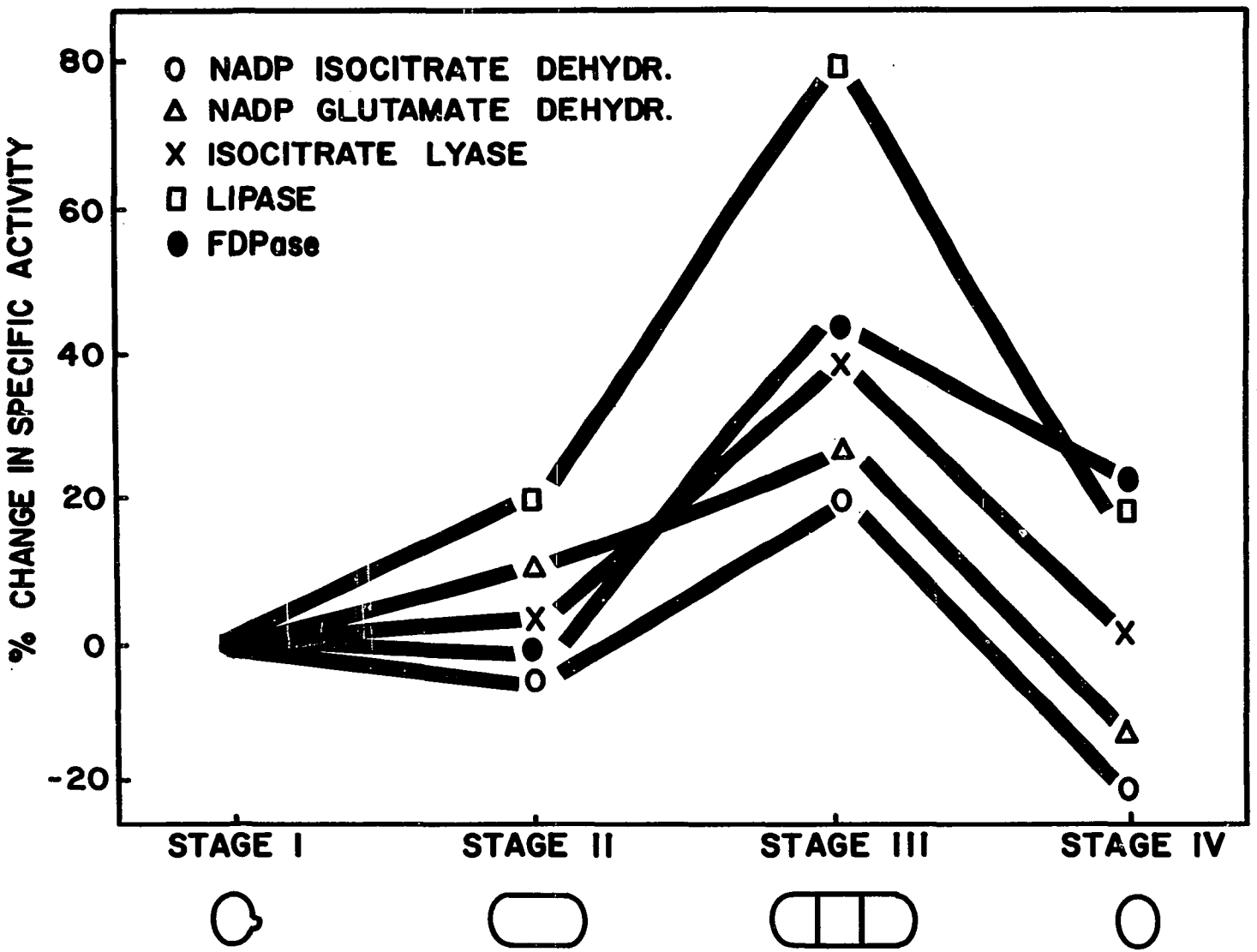

O NADP ISOCITRATE DEHYDR.
Δ NADP GLUTAMATE DEHYDR.
X ISOCITRATE LYASE
□ LIPASE
● FDPase
% CHANGE IN SPECIFIC ACTIVITY
80
60
40
20
0
-20
STAGE I
STAGE II
STAGE III
STAGE IV

Table 1.--Incorporation of D-glucose-6-C-^{14} into cells of *Arthrobacter crystallopoietes* harvested from growth on a glucose-minimal salts (GMS) medium plus succinate.[a]

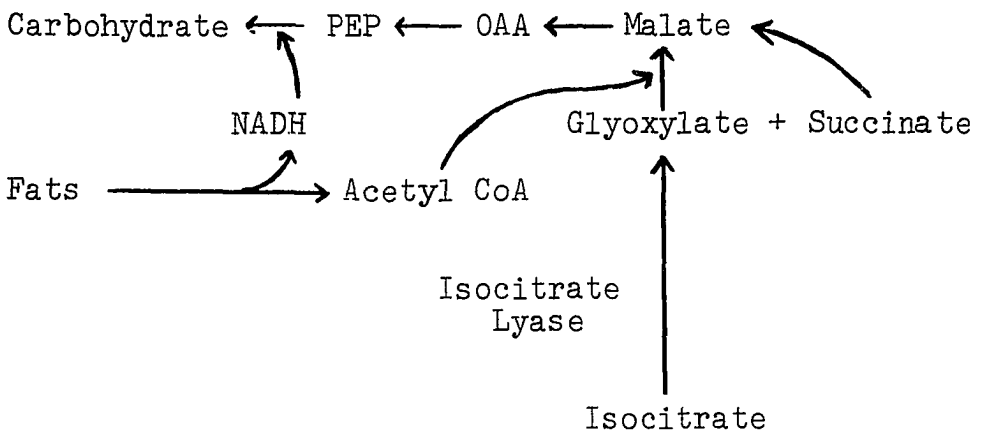
Stage of Growth	CPM of ^{14}C-glucose incorporated per ml of cells
Rod-formation (12 hr).	350
Rod-fragmentation (19 hr).	500
Coccoidal growth (30 hr)	750

[a]Cells were harvested at various stages of the life cycle and pulsed with radioactive glucose in GMS medium. Cells were shaken for 10 min at 30 C, then collected on membrane filters, washed, dried, and counted.

Krulwich and Ensign (30). In addition, Krulwich et al. (31) found that the coccoidal cell walls produced during rod-fragmentation contained much higher concentrations of carbohydrate than the cell walls of rods. Therefore it is suggested that the rate of gluconeogenesis increases during rod-fragmentation, and can thus provide the necessary carbohydrates needed for both cross-septum and coccoidal cell wall formation.

The enzyme isocitrate lyase was also found to reach its highest intracellular level during rod-fragmentation (Figure 3). Isocitrate lyase is important since it is involved in the direct conversion of fat into carbohydrate (15, 29). In addition, the enzyme has been shown to be closely associated with increased rates of gluconeogenesis (25). Isocitrate lyase is lacking in higher organisms, such as man, and, as a result, these higher organisms are unable to convert fat directly into carbohydrate (15).

Since during rod-fragmentation there is an increased rate of fat degradation accompanied by a high level of isocitrate lyase, it is believed that the isocitrate lyase can provide the necessary precursors required for an increased rate of gluconeogenesis. In addition, fat degradation can also provide both ATP and NADH necessary to drive many of the gluconeogenic reactions. The pathway described is depicted in the following diagram:



The NADP enzymes of isocitrate dehydrogenase and glutamate dehydrogenase were also found to reach their highest intracellular level during rod-fragmentation (Figure 3). Therefore it is suggested that both enzymes are also involved in gluconeogenesis, with glutamate serving as a gluconeogenic precursor.

It was also found that malate dehydrogenase increased 25% in specific activity during rod-fragmentation (Stage III), as compared to its specific activity during rod-formation (Stage II). The increase can be accounted for by the accelerated rate of gluconeogenesis during rod-fragmentation in which malate is converted to gluconeogenic OAA.

The specific activities of the glycolytic enzymes phosphofructokinase and pyruvate kinase remained essentially constant throughout morphogenesis. The specific activity of phosphofructokinase did increase in old coccoidal cells, coincidental with an observed increase in cellular permeability to exogenous glucose. Additionally, there was found

to be an increase in the specific activity of phosphoglucomutase during rod-fragmentation. This increase can be explained by the need for glucose-1-phosphate necessary for cross-septum and coccoidal cell wall formation.

Since it appears that gluconeogenesis is an active process during rod-fragmentation, the question was asked whether or not two major pathways of gluconeogenesis might exist. The first pathway would involve the conversion of OAA to phosphoenolpyruvate (PEP), by PEP carboxykinase; the second pathway would involve the conversion of pyruvate to PEP, by PEP synthase. In other organisms, both PEP carboxykinase and PEP synthase have been shown to be closely associated with increased rates of gluconeogenesis (7, 11, 43, 61). PEP carboxykinase was demonstratable in extracts of A. crystallopoietes, while PEP synthase was not demonstratable. Results similar to these had previously been shown for other species of Arthrobacter (10). From the experimental data it appears that A. crystallopoietes possesses only one gluconeogenic pathway, that of converting OAA to PEP.

From changes in specific activity data, which are probably reflections of induction or repression of enzymes, there appears to be an increased rate of lipogenesis during rod-formation, and an increased rate of gluconeogenesis during rod-fragmentation. Since malate appears to be the key intermediate in both pathways, a switch must be provided

in order to convert malate from a lipogenic pathway, to a gluconeogenic pathway. The switch can be provided, in part, by a change in specific activity of enzymes associated with the metabolism of malate. For example, the malic enzyme (lipogenic) reached its highest intracellar level during rod-formation, while the more gluconeogenic malate dehydrogenase was at a relatively low level. Thus lipogenesis was favored. On the other hand, during rod-fragmentation the situation was reversed; malate dehydrogenase was at a relatively high level, and the malic enzyme at a relatively low level. Thus gluconeogenesis was favored.

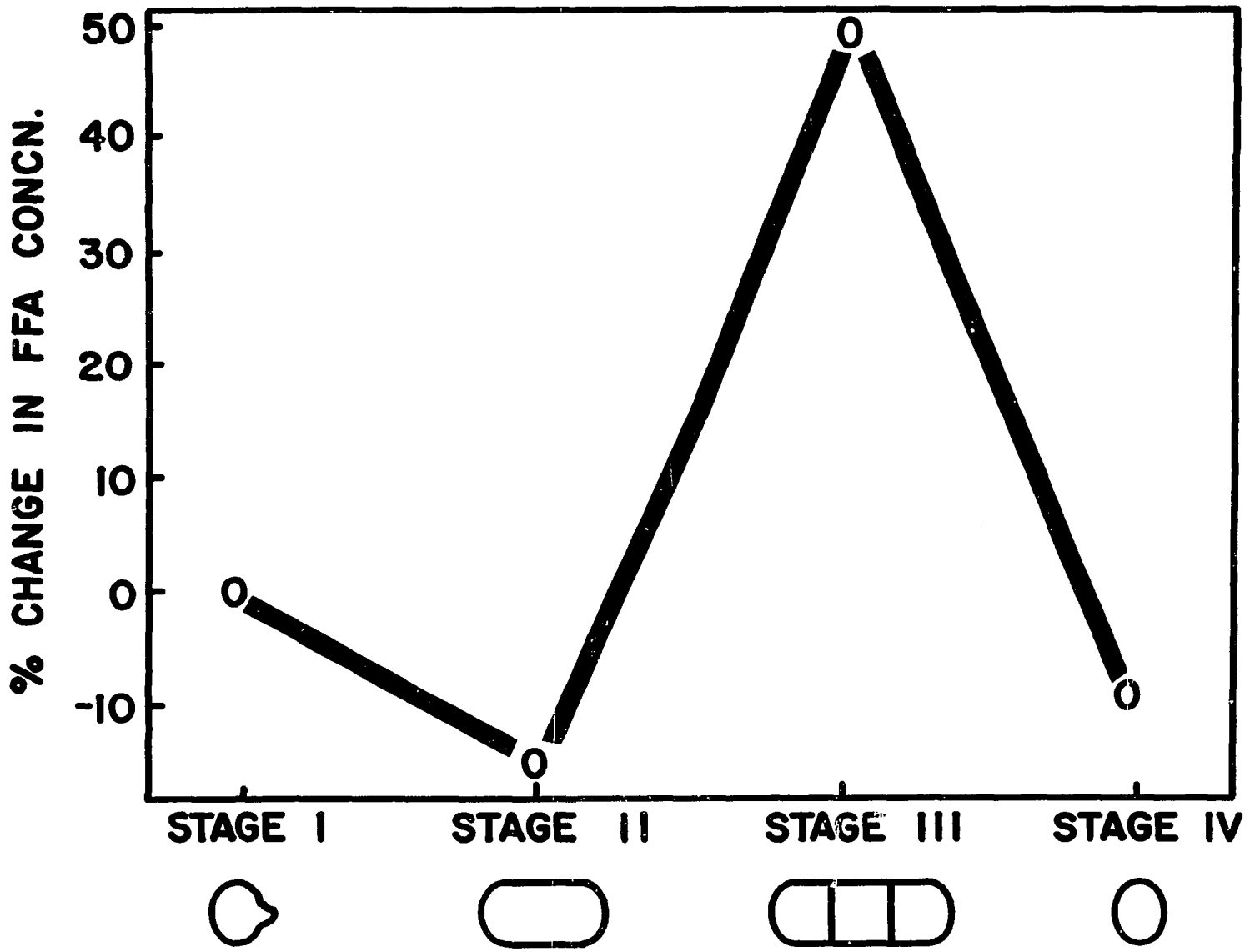
II. Effector Control of Enzymes

1. The Effect of Free Fatty Acids

The activity of enzymes can be controlled by means of small molecular weight molecules called effectors. These molecules can specifically activate or inhibit key metabolic enzymes. They have the advantage over other types of regulation in that their effect can be mediated almost instantaneously.

Upon examination of the intracellular free fatty acid (FFA) pool of A. crystallopoietes, it was found that the relative concentration of FFA reached its highest intracellular level during rod-fragmentation (Figure 4), coincidental with the increased rate of gluconeogenesis. Therefore the question was asked whether or not, FFA, in addition to providing carbohydrate precursors for rod-fragmentation, could

Fig. 4.--Per cent change in the concentration of intracellular free fatty acids as a function of the growth cycle of Arthrobacter crystallopoietes.


% CHANGE IN FFA CONCN.
50
40
30
20
10
0
-10
STAGE I
STAGE II
STAGE III
STAGE IV

also stimulate the conversion of fat into carbohydrate through allosteric regulation.

From *in vitro* experiments it was found that FFA could selectively inhibit those enzymes of *A. crystallopoietes* which were involved in lipogenesis, or opposed to gluconeogenesis (Table 2), but not inhibit those enzymes involved in non-lipogenic processes. Previously it was demonstrated in other organisms that active lipolysis was accompanied by an increased release of FFA (40). This resulted in a reduced flux of glucose through the glycolytic pathway, and a marked stimulation of gluconeogenesis (20, 46, 53).

In *A. crystallopoietes*, FFA were found to competitively inhibit the glycolytic enzymes, phosphofructokinase, as shown in Figure 5, and pyruvate kinase, as shown in Figures 6 and 7. The degree of pyruvate kinase inhibition by FFA, in the presence of substrates PEP and ADP, is emphasized in the velocity versus substrate plots of Figures 8 and 9 respectively.

The selective nature of FFA inhibition was demonstrated by the fact that such gluconeogenic enzymes as fructose-1,6-diphosphatase were not inhibited by FFA. Additionally, such bifunctional enzymes as phosphohexoisomerase, functioning both in glycolysis and gluconeogenesis, and phosphoglucomutase, were also not inhibited by FFA. Thus it is suggested that FFA play an important role in the

Table 2.--Per cent inhibition of enzyme activities of *Arthrobacter crystallopoietes* in the presence of 33 mM sodium octanoate, 2.5 mM sodium laurate, and 0.67 mM sodium myristate. Km values were used for respective substrate concentrations while all coenzyme concentrations were saturating.

Enzyme	Substrate	Octanoate	Laurate	Myristate
Phosphofructokinase	fructose-6-phosphate	45	a	a
Glucose-6-phosphate dehydrogenase	glucose-6-phosphate	58	a	a
Malic enzyme	malate	30	a	a
Pyruvate kinase	ADP	100	a	a
	PEP	58	a	a
Lactate dehydrogenase	pyruvate	58	100	a
Fumarase	malate	60	45	68

[a]Precipitation in the reaction mixture precluded analysis.

Fig. 5.--Double reciprocal plot of velocity *versus* fructose-6-phosphate concentration for phosphofructokinase. Assay conditions described in methods. O, no inhibitor; Δ, 33 mM sodium octanoate.

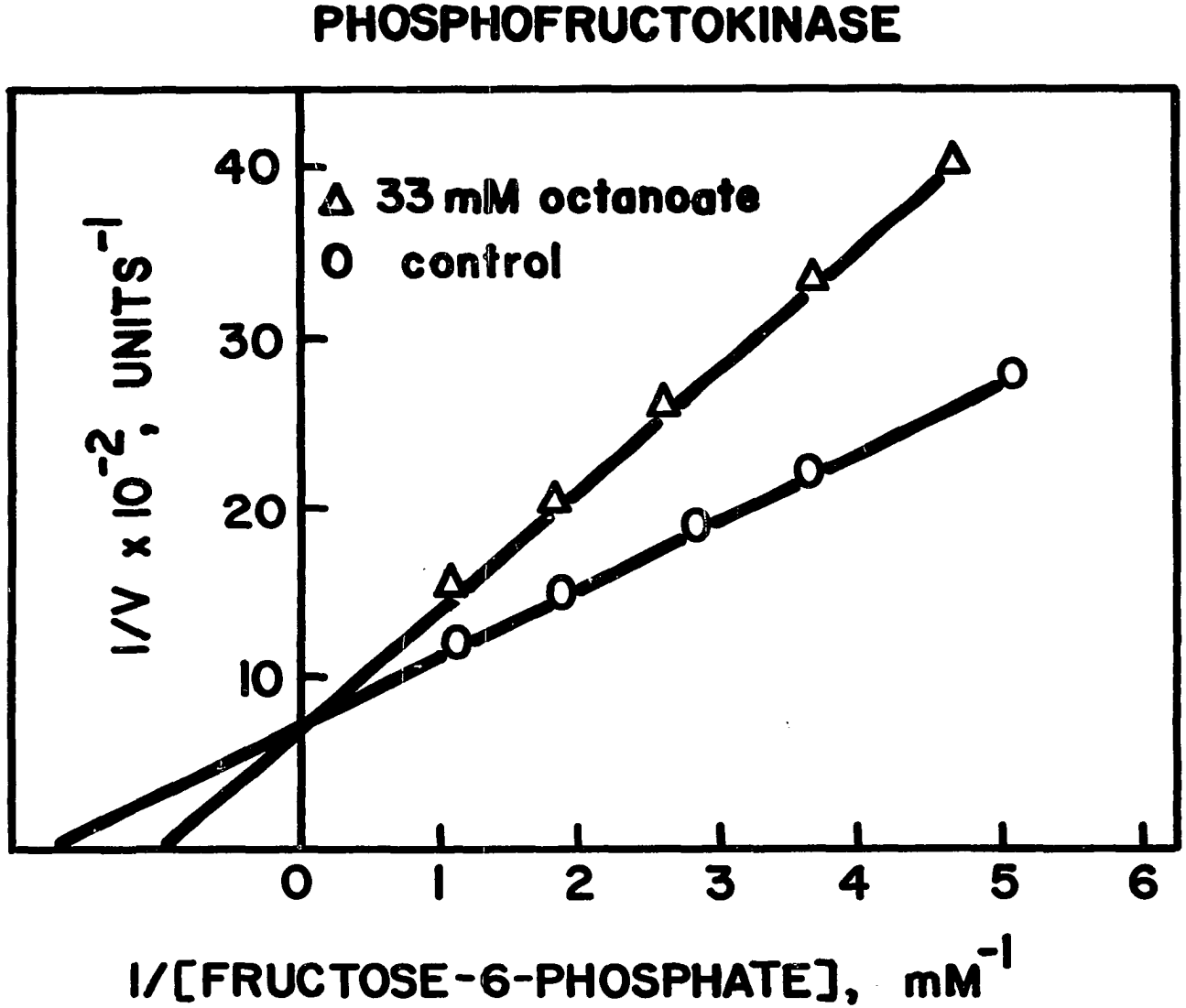

PHOSPHOFRUCTOKINASE
40
30
20
10
Δ 33 mM octanoate
O control
1/V x 10^-2, UNITS^-1
0
1
2
3
4
5
6
1/[FRUCTOSE-6-PHOSPHATE], mM^-1

Fig. 6.--Double reciprocal plot of velocity *versus* phosphoenolpyruvate concentration for pyruvate kinase. Assay conditions described in methods. **o**, no inhibitor; **Δ**, 33 mM sodium octanoate.

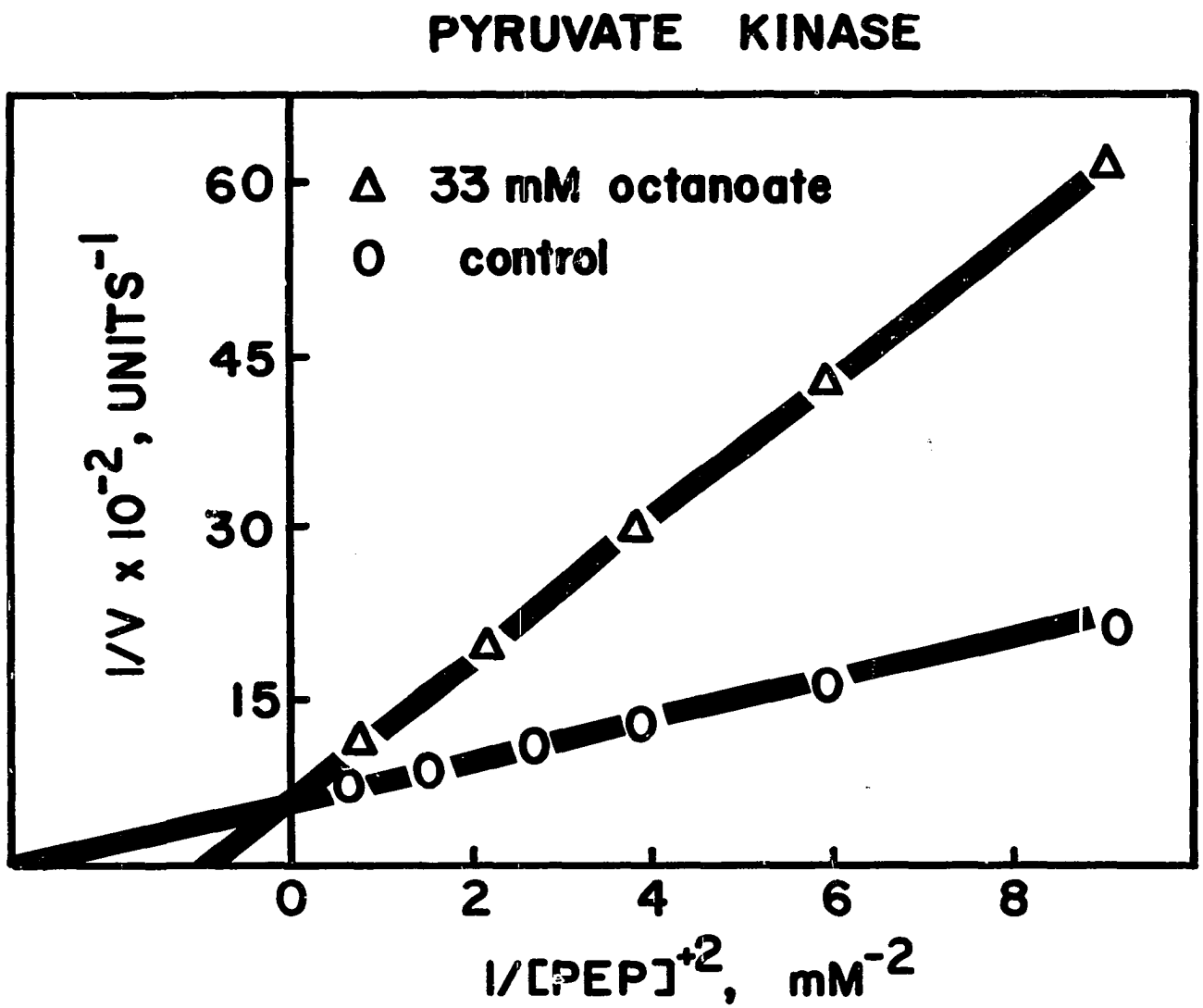

PYRUVATE KINASE
Δ 33 mM octanoate
0 control
60
45
30
15
1/V x 10⁻², UNITS⁻¹
0
2
4
6
8
1/[PEP]⁺², mM⁻²

Fig. 7.--Double reciprocal plot of velocity *versus* ADP concentration for pyruvate kinase. Assay conditions described in methods. O, no inhibitor; Δ, 33 mM sodium octanoate.

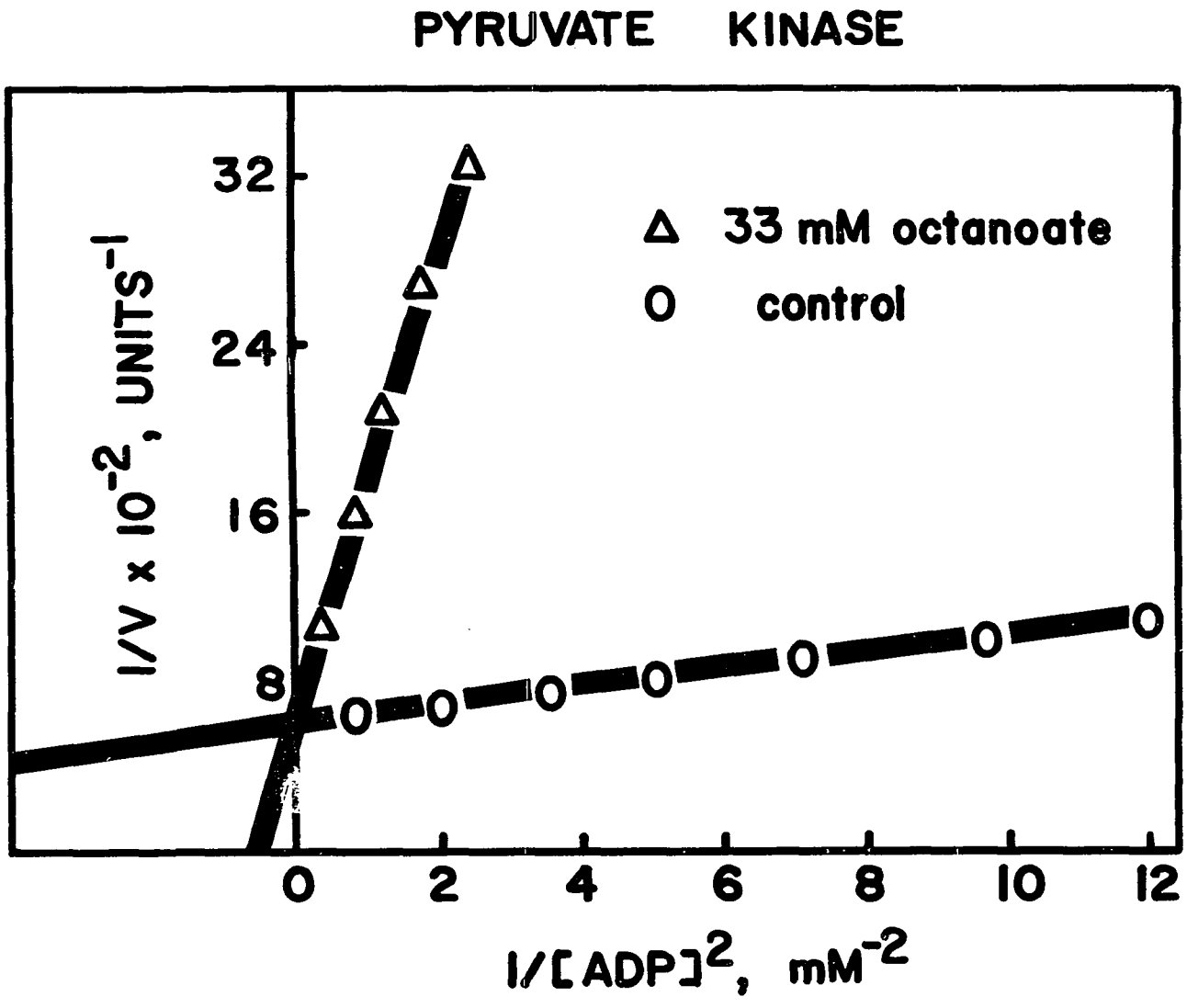

PYRUVATE KINASE
32
24
16
8
1/V x 10^-2, UNITS^-1
Δ 33 mM octanoate
O control
0
2
4
6
8
10
12
1/[ADP]^2, mM^-2

Fig. 8.--A plot of velocity (units) as a function of phosphoenolpyruvate concentration for pyruvate kinase. Assay conditions described in methods. o, no inhibitor; Δ, 33 mM sodium octanoate.

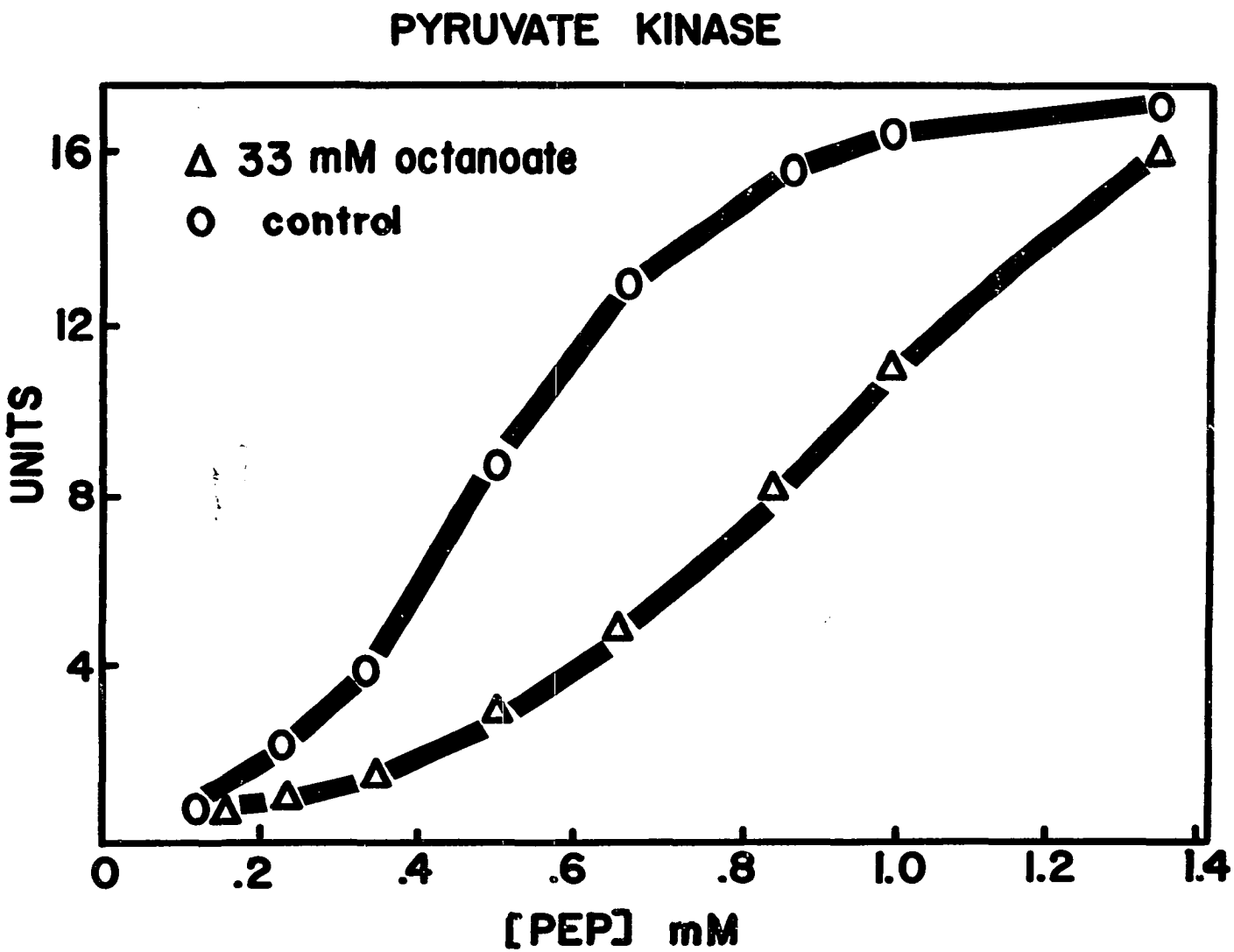
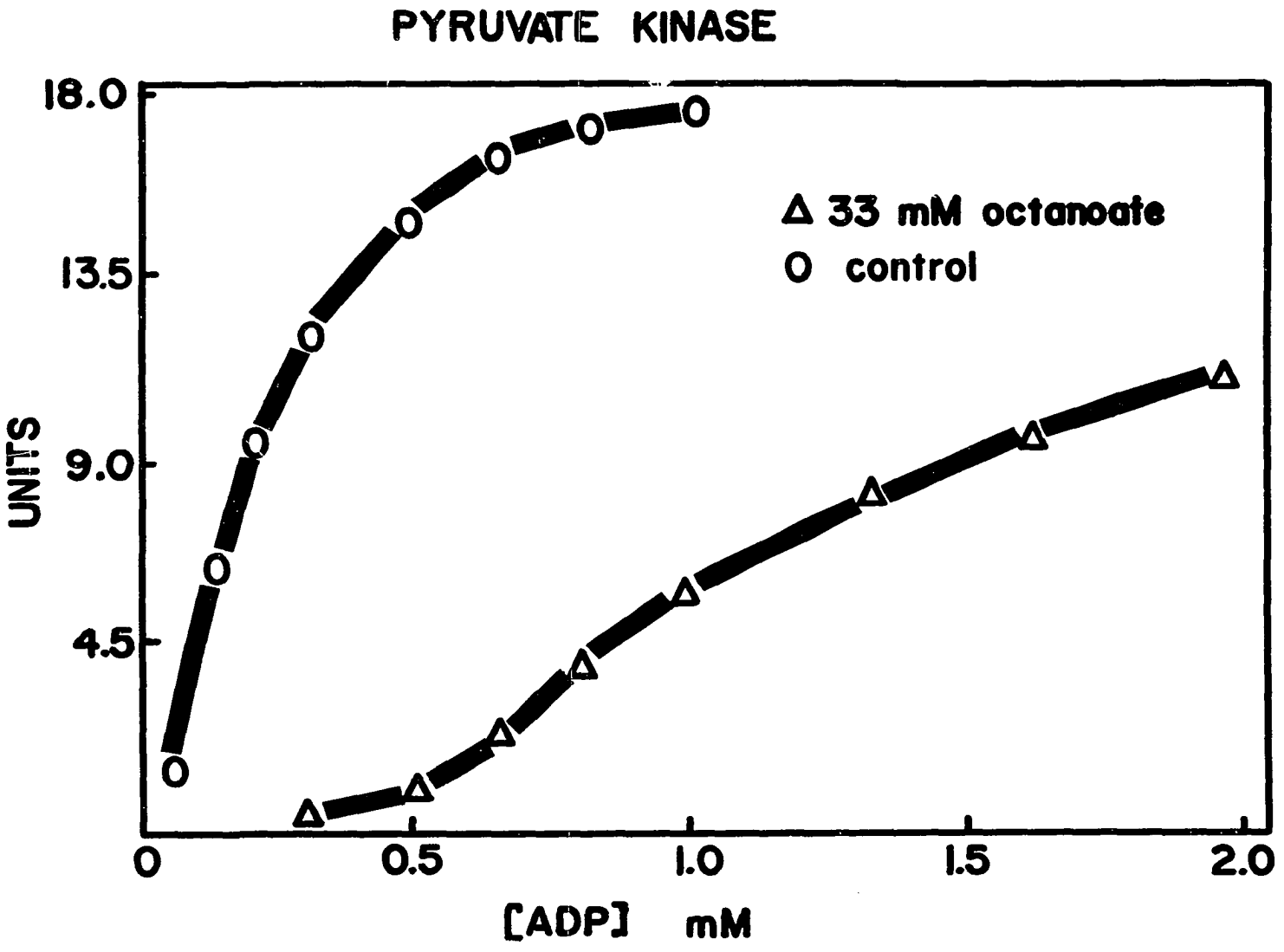

PYRUVATE KINASE
Δ 33 mM octanoate
O control
UNITS
16
12
8
4
0
.2
.4
.6
.8
1.0
1.2
1.4
[PEP] mM

Fig. 9.--A plot of velocity (units) as a function of ADP concentration for pyruvate kinase. Assay conditions described in methods. O, no inhibitor; Δ, 33 mM sodium octanoate.


PYRUVATE KINASE
18.0
13.5
9.0
4.5
UNITS
Δ 33 mM octanoate
O control
0
0.5
1.0
1.5
2.0
[ADP] mM

regulation of both glycolysis and gluconeogenesis of A. crystallopoietes.

FFA were also found to inhibit competitively both the malic enzyme (Figure 10), and glucose-6-phosphate dehydrogenase (Figure 11). This was expected from the fact that both enzymes can produce NADPH, and, as previously mentioned, have a marked correlation with increased lipogenesis. Furthermore, inhibition of the malic enzyme by FFA, and lack of inhibition of malate dehydrogenase by FFA, provides another switch for shifting malate from a lipogenic pathway to a gluconeogenic pathway.

NADP isocitrate dehydrogenase, which can also produce NADPH, was not inhibited by FFA. From specific activity data (Figure 3) the enzyme appears to be involved in gluconeogenesis, rather than lipogenesis. Furthermore, in other organisms NADP isocitrate dehydrogenase had been shown to have very little correlation with increased lipogenesis (21, 37).

FFA did not inhibit the first enzyme of the glyoxylate pathway, isocitrate lyase. This was expected from the fact that isocitrate lyase is involved in the conversion of fat into carbohydrate, and can thus play an important role in gluconeogenesis (15). Furthermore, since the glyoxylate pathway does utilize acetyl CoA, and does compete with a lipogenic pathway for acetyl CoA (15), isocitrate lyase inhibition would not have been expected.

Fig. 10.--Double reciprocal plot of velocity *versus* malate concentration for the malic enzyme. Assay conditions described in methods. ○, no inhibitor; △, 16.7 mM sodium octanoate.

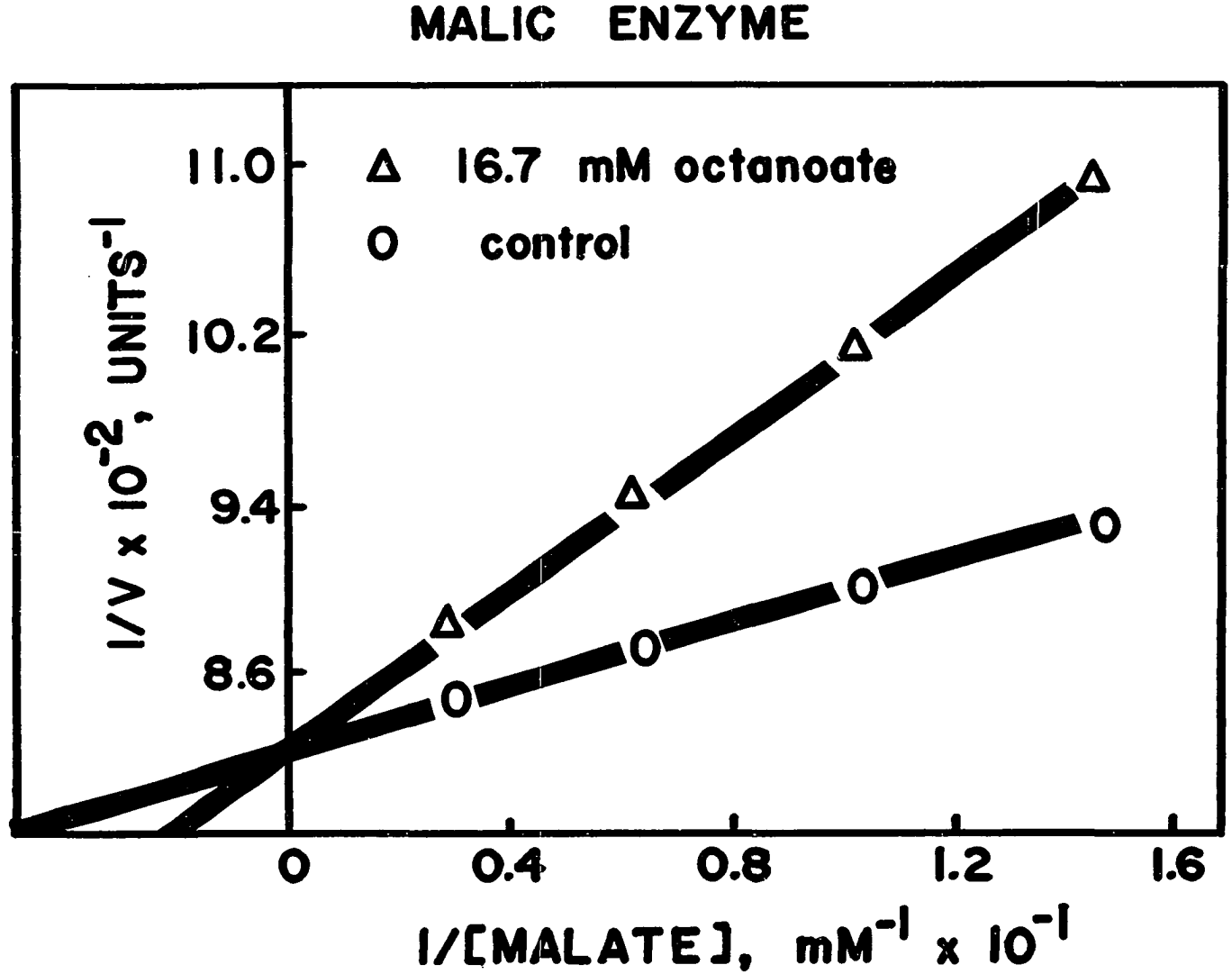
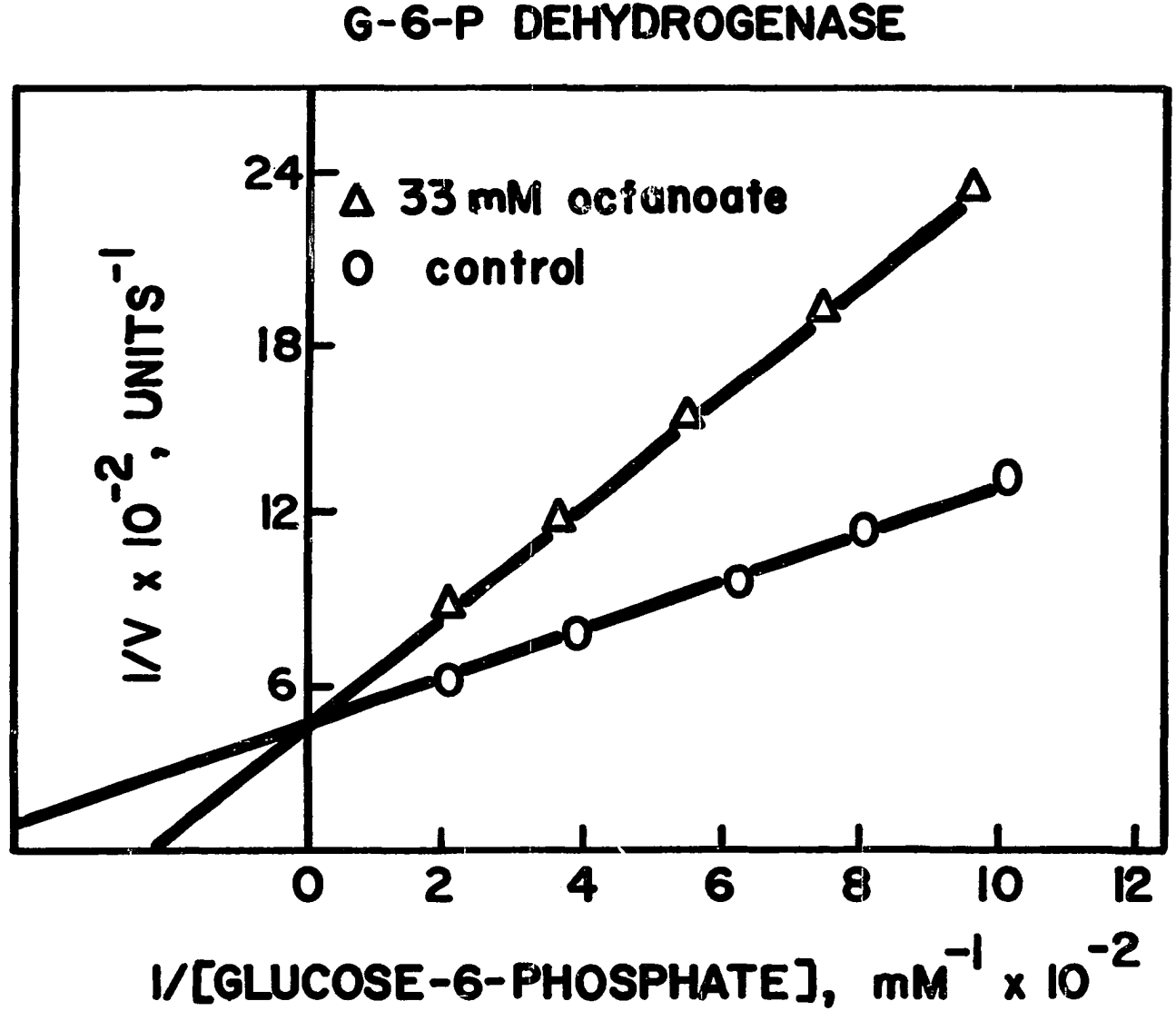

MALIC ENZYME
11.0
10.2
9.4
8.6
Δ 16.7 mM octanoate
O control
1/V x 10^-2, UNITS^-1
0
0.4
0.8
1.2
1.6
1/[MALATE], mM^-1 x 10^-1

Fig. 11.--Double reciprocal plot of velocity *versus* glucose-6-phosphate concentration for glucose-6-phosphate dehydrogenase. Assay conditions described in methods. **o**, no inhibitor; **Δ**, 33 MM sodium octanoate.

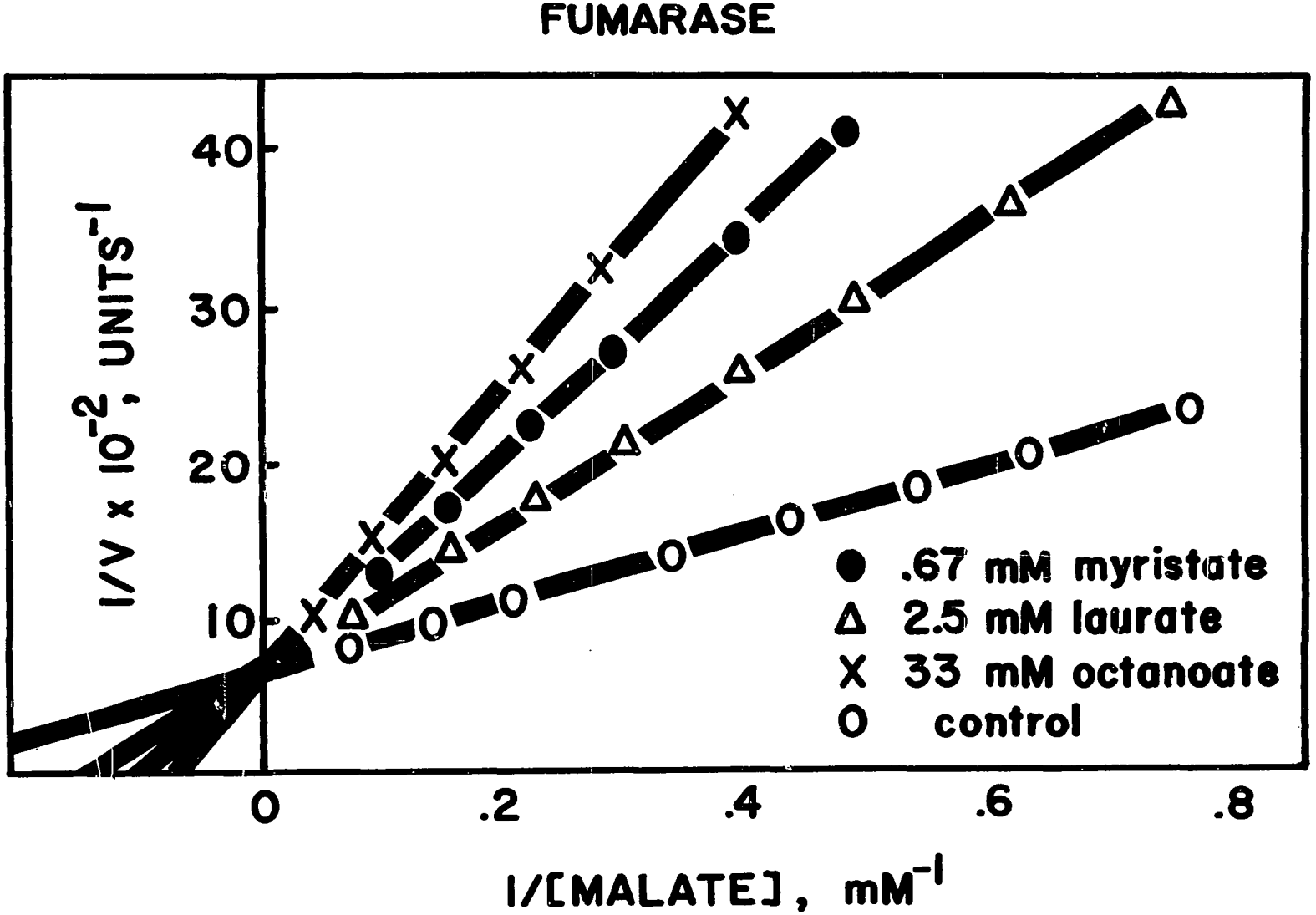

G-6-P DEHYDROGENASE
Δ 33 mM octanoate
O control
1/V x 10^-2, UNITS^-1
24
18
12
6
0
2
4
6
8
10
12
1/[GLUCOSE-6-PHOSPHATE], mM^-1 x 10^-2

Other enzymes not inhibited by FFA were aconitase and NADP glutamate dehydrogenase. From specific activity data, NADP glutamate dehydrogenase appears to be involved in gluconeogenesis (Figure 3).

It was also found that the Krebs cycle enzyme fumarase was competitively inhibited by FFA (Figure 12). An explanation for the inhibition was provided by Weber et al. (55), in which it was stated that inhibition of fumarase could prevent a "leaking back" of malate into fumarate. This in turn would allow malate a chance to stimulate gluconeogenesis by providing an available source of OAA.

During the FFA inhibition studies, it was found that the longer chain FFA of sodium laurate and sodium myristate, could bring about enzyme inhibition at a much lower molar concentration than the shorter chain FFA, sodium octanoate (Table 2). Thus, in vivo, the longer chain FFA may be the actual mediator of the effect of selective enzyme inhibition. Sodium laurate and sodium myristate were omitted from inhibition studies in which Mg^{++} or Mn^{++} were required for enzyme activity. This was due to the fact that a precipitate was formed between metal ion and FFA which prevented any quantitation of enzyme reaction. Therefore sodium octanoate (short chain FFA) was used for most of the FFA inhibition studies. It must be noted that even though the concentration of octanoate used in vitro was relatively high, this

Fig. 12.--Double reciprocal plot of velocity versus malate concentration for fumarase. Assay conditions described in methods. O, no inhibitor; ●, 33 mM sodium octanoate; Δ, 2.5 mM sodium laurate; ×, 0.67 mM sodium myristate.


FUMARASE
40
30
20
10
1/V x 10^-2; UNITS^-1
0
.2
.4
.6
.8
1/[MALATE], mM^-1
.67 mM myristate
2.5 mM laurate
33 mM octanoate
control

concentration could easily be achieved by assuming compartmentation within the bacterial cell.

All enzymes, except one, which were selectively inhibited by FFA, were inhibited in a competitive manner. The exception was lactate dehydrogenase which was inhibited in a non-competitive manner by FFA (Table 3). The K_I values of lactate dehydrogenase for FFA were about 10-fold higher than the K_I values calculated for other FFA inhibited enzymes (Table 3). Therefore, FFA inhibition of lactate dehydrogenase may be the result of non-specific detergent effect, and not the result of selective enzyme inhibition.

A crude lipid extract obtained from washed cells of A. crystallopoietes was found to produce essentially the same type of selective inhibition of bacterial enzymes as did FFA. This experiment was performed only to demonstrate that a naturally occurring lipid product from a bacterial cell could be of some physiological significance as a possible control mechanism. No attempt was made to quantitate the extract or to determine its composition.

From the experimental data it is suggested that FFA can play an important role in the control of morphogenesis of A. crystalloporetes through the regulation of the activity of enzymes involved in the metabolism of fats and carbohydrates.

Table 3. Kinetic data obtained from enzymes of Arthrobacter crystallopoietes inhibited by FFA. Values for Km and K_I of enzymes and the type of inhibition [competitive (C) or non-competitive (NC)] produced by the effect of FFA are obtained from Lineweaver-Burk plots.[a]

Enzymes	Substrate	Km	Octanoate K_I	Laurate K_I	Myristate K_I
		mM		mM	
Phosphofructokinase	fructose-6-phosphate	1.0	20.0(C)	b	b
Glucose-6-phosphate dehydrogenase	glucose-6-phosphate	3.4	36.0(C)	b	b
Malic enzyme	malate	0.60	6.7(C)	b	b
Pyruvate kinase	ADP	0.22	3.7(C)	b	b
Lactate dehydrogenase	pyruvate	28.0	200.0 (NC)	24.0 (NC)	b
Fumarase	malate	2.0	23.0(C)	1.8(C)	0.16 (C)

[a]FFA concentrations employed were 33 mM sodium octanoate, 2.5 mM sodium laurate, and 0.67 mM sodium myristate. For lactate dehydrogenase 0.67 mM sodium laurate was used instead of 2.5 mM concentration.

[b]Precipitation in the reaction mixture precluded analysis.

2. Implications of Glutamate as a Gluconeogenic Precursor

From specific activity data it was found that NADP glutamate dehydrogenase reached its highest intracellular level during rod-fragmentation, coincidental with the time of an apparent increase in gluconeogenesis. Previously it had been suggested by Wright (58, 59), that during the morphogenesis of the slime mold Dictyostelium discoideum, that glutamate was converted to carbohydrate via glutamate dehydrogenase. Furthermore, for other organisms, both glutamate and glutamate dehydrogenase had been shown to be closely associated with increased gluconeogenesis (16, 53).

The NADP glutamate dehydrogenase isolated from A. crystallopoietes was found to have an extremely high K_m for substrate glutamate (75 mM). In order for the enzyme to convert glutamate to α-ketoglutarate in vivo, an extremely high intracellular concentration of glutamate would be required. From an investigation of the free amino acid pool of A. crystallopoietes, this requirement appears to met in that high concentrations of glutamate were found to be present at all stages of growth. In fact over 95% of the entire free amino acid pool appeared to be present as glutamate. In addition, the NADP glutamate dehydrogenase isolated from N. corallina was also found to have an extremely high K_m for glutamate (80 mM). Furthermore, as was found to be the case for Arthrobacter, Hittle (24) found in N. corallina an extremely large quantity of glutamate in the

free amino acid pool. As previously mentioned, both organisms do undergo sphere-rod morphogenesis.

From kinetic experiments as shown in Figures 13 and 14, it was found that FFA specifically activated glutamate dehydrogenase by lowering the apparent K_m of the enzyme for substrate glutamate. In addition, it was found that FFA also decreased the substrate binding site interaction of the enzyme as shown by the Hill plot in Figure 15. Davis and Gibson (16) showed in the perfused rat liver that fatty acids could stimulate liver glutamate dehydrogenase activity. The proposed mechanism was an uncoupling of oxidative phosphorylation by fatty acids, which in turn produce an increased concentration of substrate NADP. The explanation seems incomplete in that no experiments were carried out to test the direct effect of fatty acids on the enzyme itself.

From the high levels of glutamate, glutamate dehydrogenase, and FFA found to be present at the time of rod-fragmentation, and the fact that FFA can stimulate glutamate dehydrogenase activity, it is suggested that both glutamate and glutamate dehydrogenase are involved in active gluconeogenesis during rod-fragmentation of A. crystallopoietes.

It is also suggested that the combined action of NADP glutamate dehydrogenase, and NADP isocitrate dehydrogenase, can stimulate gluconeogenesis during rod-fragmentation by catalyzing the conversion of glutamate to α-ketoglutarate, followed by conversion to isocitrate. The

Fig. 13.--A plot of velocity (units) as a function of glutamate concentration for NADP glutamate dehydrogenase. Assay conditions described in methods. o, no activator; Δ, 33 mM sodium octanoate.

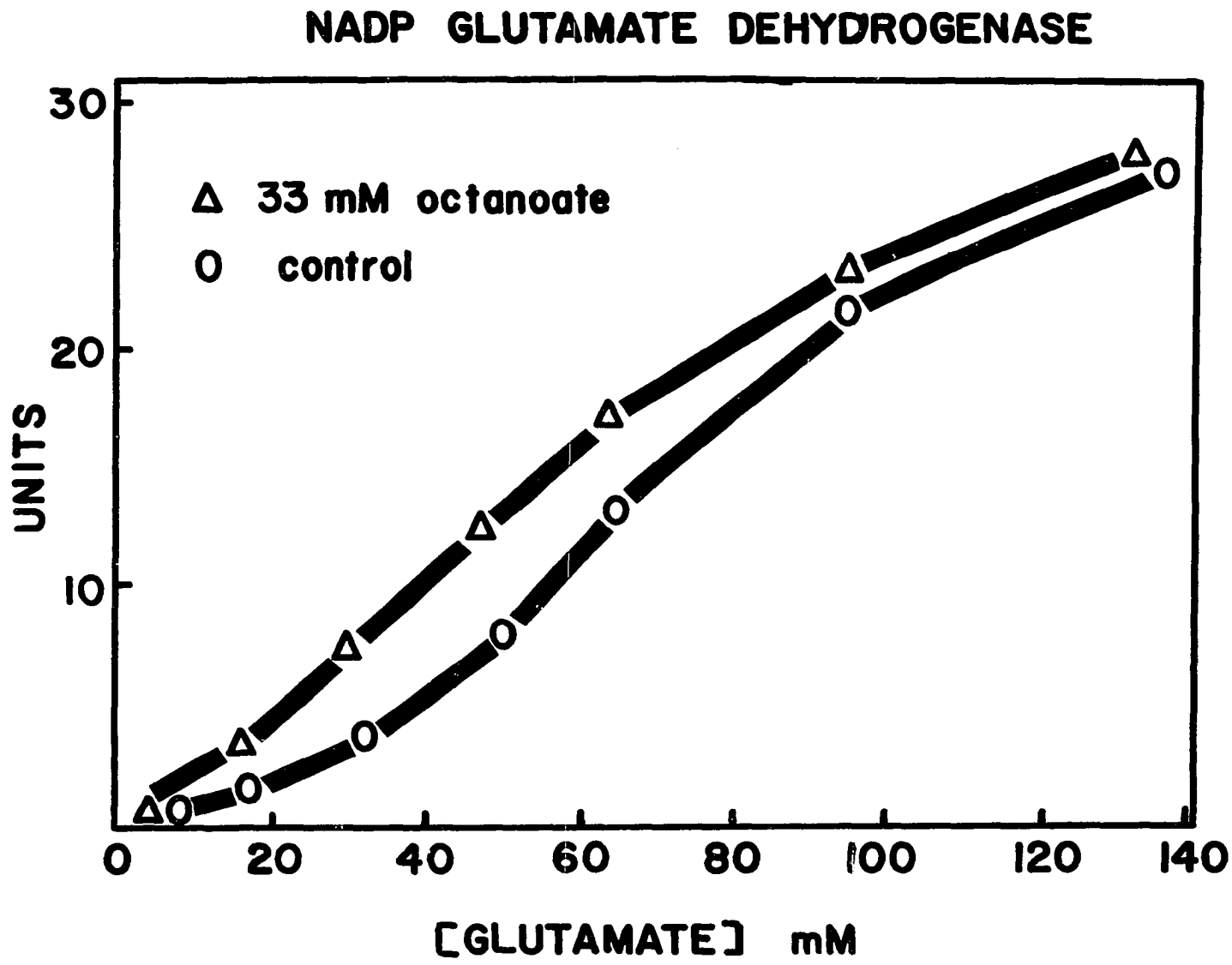

NADP GLUTAMATE DEHYDROGENASE
Δ 33 mM octanoate
O control
UNITS
30
20
10
0
20
40
60
80
100
120
140
[GLUTAMATE] mM

Fig. 14.--Double reciprocal plot of velocity *versus* glutamate concentration for NADP glutamate dehydrogenase. Assay conditions described in methods. ○, no activator; △, 33 mM sodium octanoate.

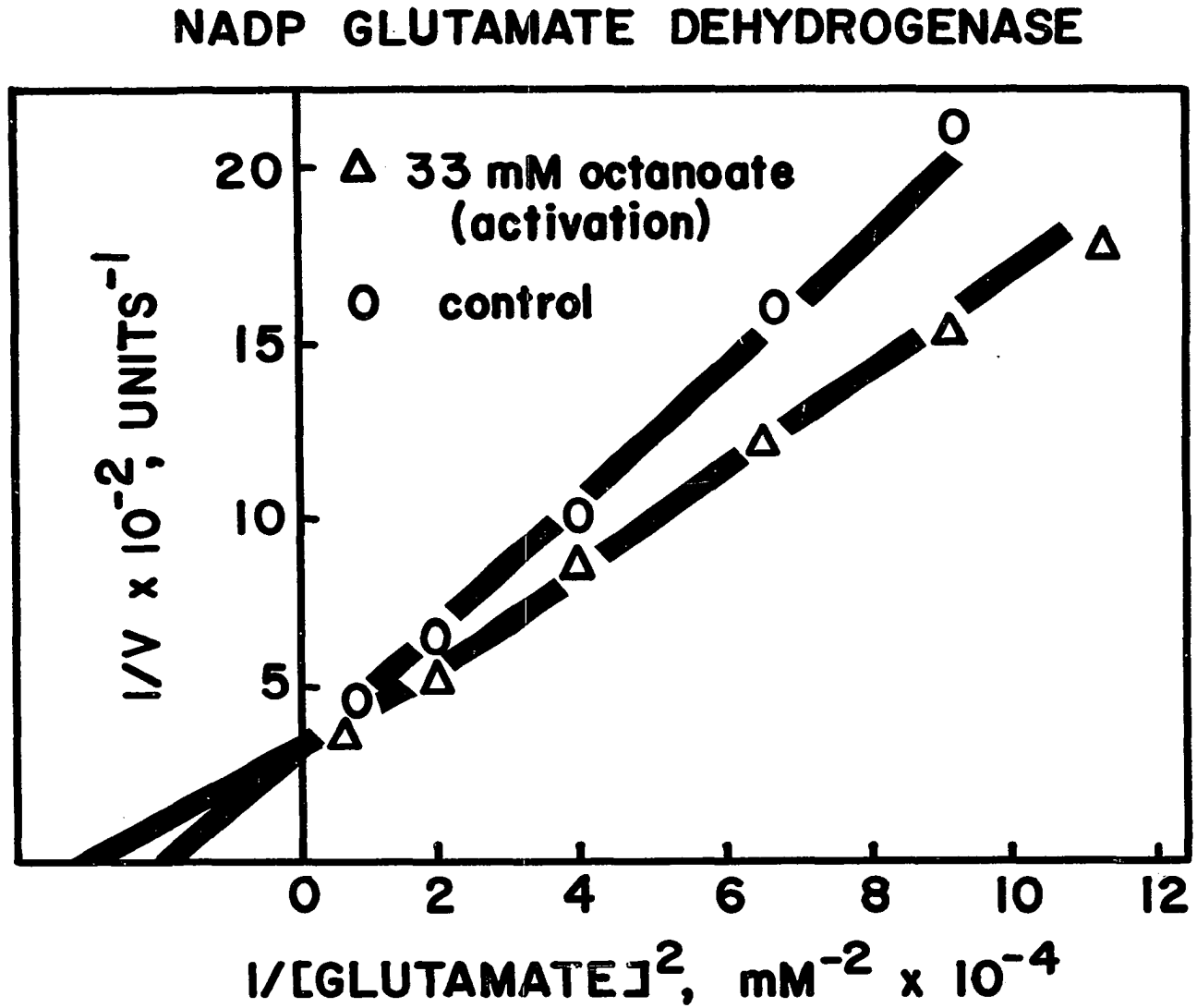
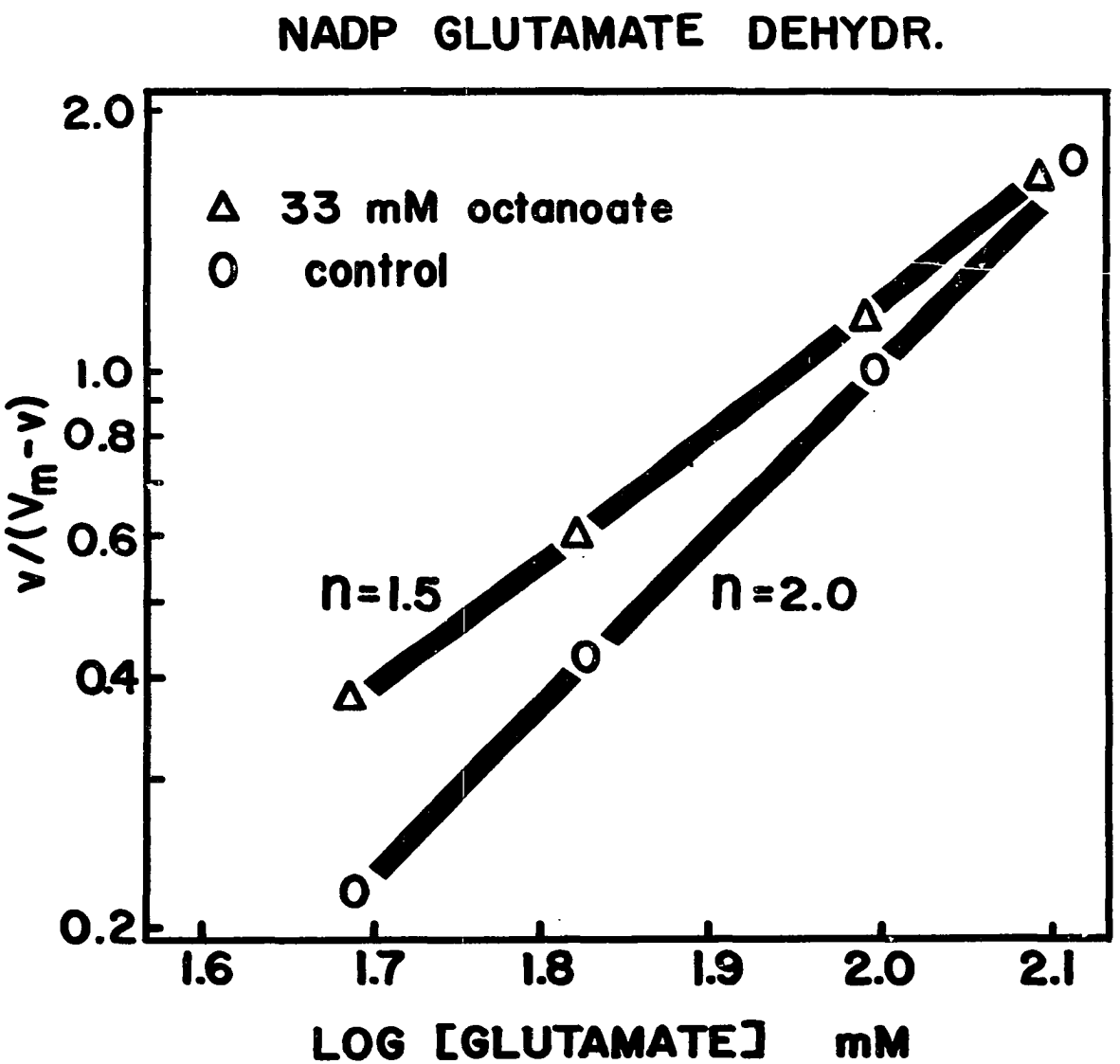

NADP GLUTAMATE DEHYDROGENASE
Δ 33 mM octanoate (activation)
O control
1/V x 10^-2, UNITS^-1
20
15
10
5
0
2
4
6
8
10
12
1/[GLUTAMATE]^2, mM^-2 x 10^-4

Fig. 15.--Hill plot of NADP glutamate dehydrogenase with respect to substrate glutamate. A slope or interaction coefficient (n) of 2.0 was obtained in the absence of sodium octanoate, but decreased to 1.5 in the presence of 33 mM sodium octanoate. Assay conditions described in methods. O, no activator; Δ, 33 mM sodium octanoate.


NADP GLUTAMATE DEHYDR.
2.0
1.0
0.8
0.6
0.4
0.2
v/(Vm-v)
Δ 33 mM octanoate
O control
n=1.5
n=2.0
1.6
1.7
1.8
1.9
2.0
2.1
LOG [GLUTAMATE] mM

resulting isocitrate can then be channeled through the glyoxylate pathway to carbohydrate. The resulting gluconeogenic sequence would be: glutamate ⟶ α-ketoglutarate ⟶ isocitrate ⟶ glyoxylate pathway ⟶ carbohydrate.

Only the NADP dependent glutamate and isocitrate dehydrogenases have been detected in extracts of A. crystallopoietes. No NAD dependent glutamate and isocitrate dehydrogenases have been detected. The NADP dependent isocitrate dehydrogenase can catalyze the conversion of α-ketoglutarate to isocitrate (the gluconeogenic direction), a conversion not possible with the NAD dependent enzyme (38). In addition the NAD dependent isocitrate dehydrogenase isolated from other organisms has been found to be under adenylate control, and shown to play an important role in the regulation of energy metabolism (2). The NADP dependent isocitrate dehydrogenase isolated from A. crystallopoietes was found not to be affected by either ATP, ADP, or AMP in concentrations up to 10 mM. This suggests that the NADP dependent isocitrate dehydrogenase of A. crystallopoietes is not involved in the regulation of energy metabolism.

Since NADP isocitrate dehydrogenase reached its highest intracellular level during rod-fragmentation, and was not inhibited by FFA, it is believed that the enzyme does play an active role in gluconeogenesis during rod-fragmentation. The idea is also supported by the studies of inhibition of isocitrate dehydrogenase by OAA and

glyoxylate. It was found that 10 mM of OAA or glyoxylate, separately, would not inhibit isocitrate formation from α-ketoglutarate (gluconeogenic direction), but when mixed together in concentrations of 0.67 mM OAA, and 0.67 mM glyoxylate, would competitively inhibit the enzyme in a concerted or synergistic fashion (Figure 16). The calculated K_I value for the concerted inhibition was 1.1 mM. Therefore OAA and glyoxylate acting together synergistically could become a feedback inhibitor of both isocitrate formation and gluconeogenesis.

For the reverse reaction, formation of α-ketoglutarate from isocitrate (non-gluconeogenic direction), it was found that OAA or glyoxylate, separately, in concentrations up to 3 mM, would not inhibit enzyme activity. Yet when the two compounds were mixed in concentrations as small as 0.125 mM OAA, and 0.125 mM glyoxylate, a concerted type of competitive inhibition was found to occur (Figure 17). The calculated K_I for the inhibition was 5.5 μM, considerably smaller than the calculated K_I value of 1.1 mM for the reverse reaction.

Therefore at relatively low concentrations of glyoxylate and OAA, the reaction is inhibited in the non-gluconeogenic direction (α-ketoglutarate formation), whereas the reaction in the gluconeogenic direction (isocitrate formation) is not inhibited. Since low concentrations of glyoxylate and OAA would be present during the initiation of gluoconeogenesis, it would be desirable to inhibit the

Fig. 16.--Double reciprocal plot of velocity versus α-ketoglutarate concentration for NADP isocitrate dehydrogenase. Assay conditions described in methods. ○, no inhibitor; △, 0.67 mM glyoxylate plus 0.67 mM oxalacetate. Separately, 10 mM of glyoxylate or oxalacetate produced no inhibition of enzyme activity.

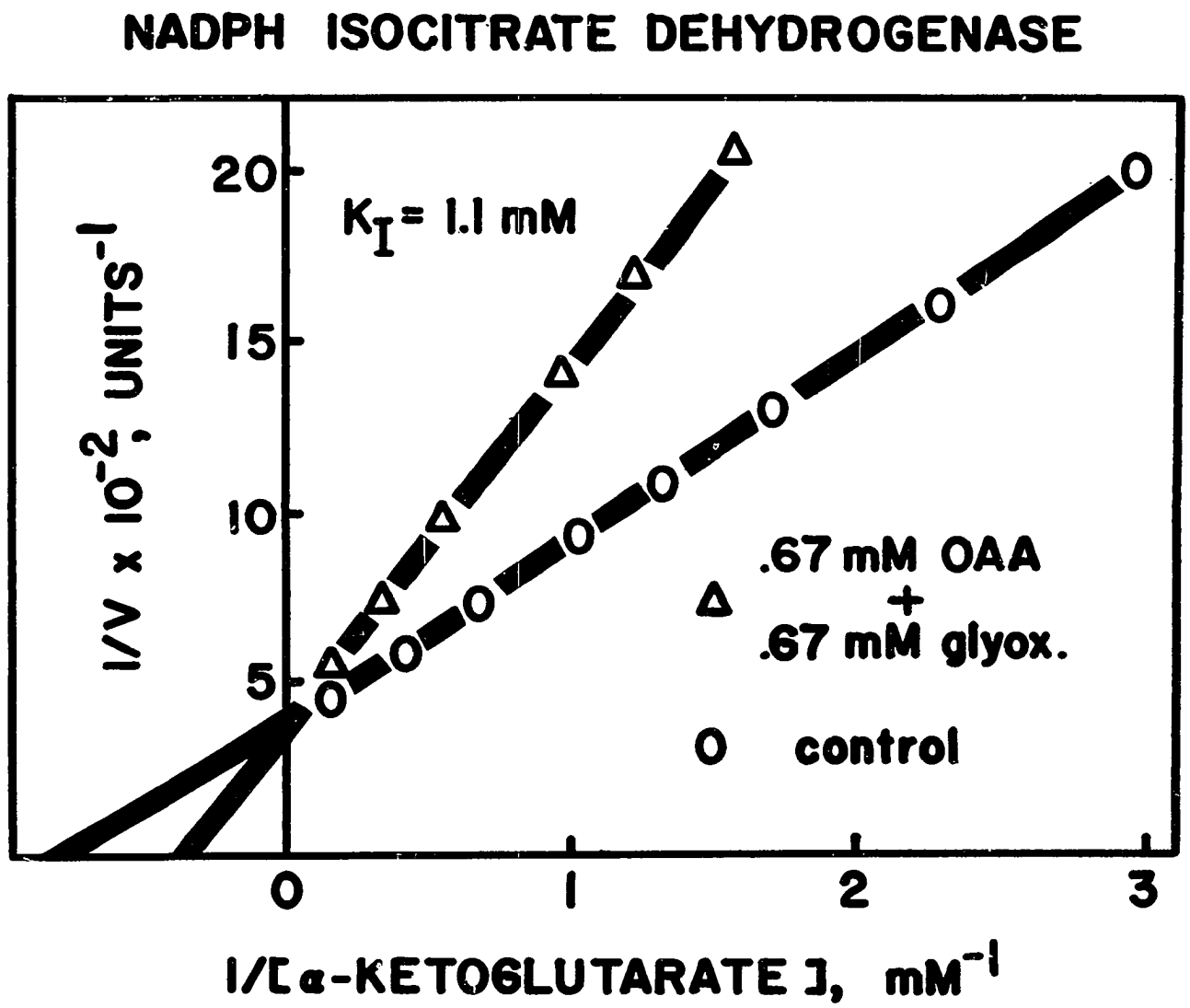
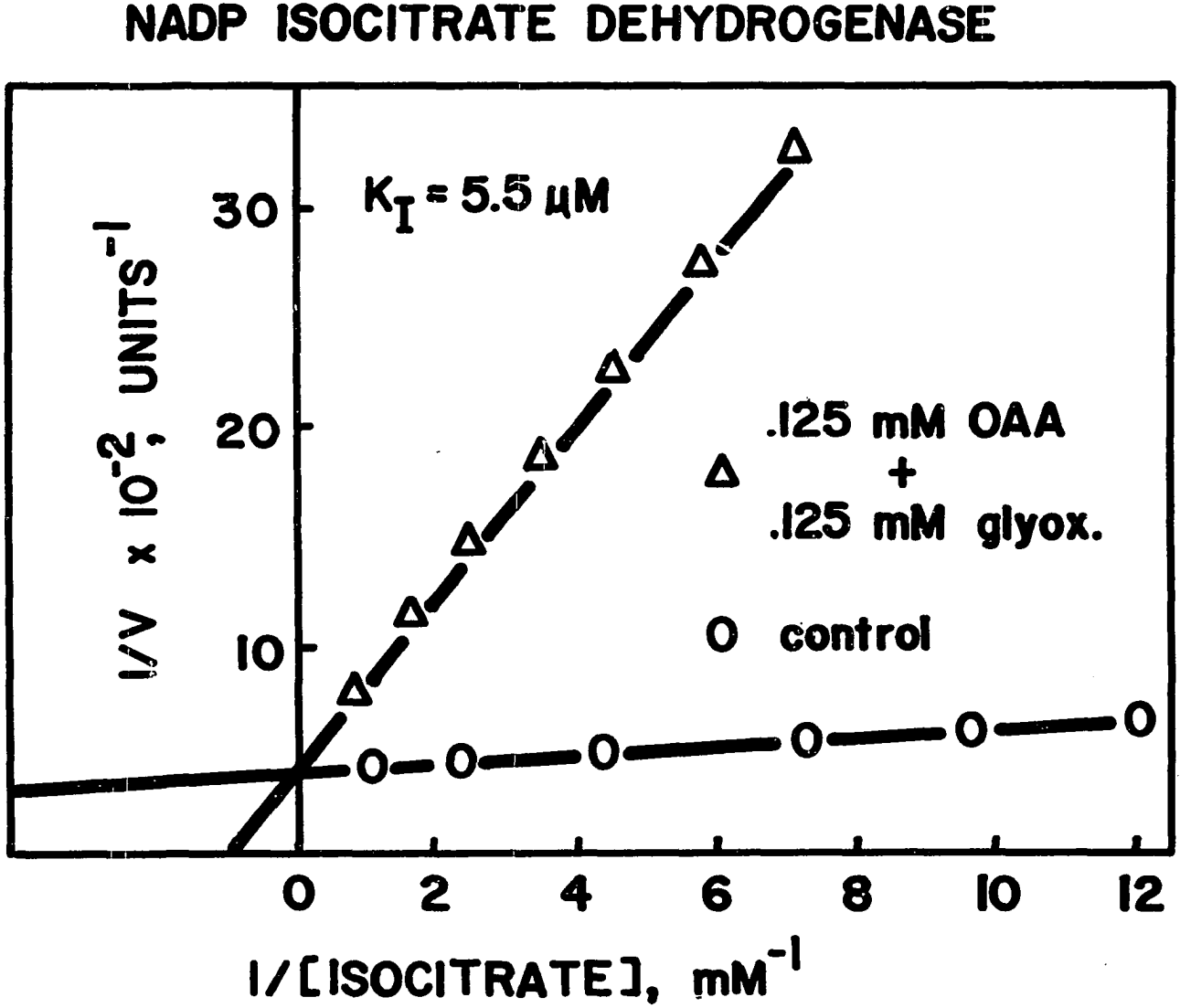

NADPH ISOCITRATE DEHYDROGENASE
K_I = 1.1 mM
.67 mM OAA
+
.67 mM glyox.
control
1/V x 10^{-2}, UNITS^{-1}
1/[α-KETOGLUTARATE], mM^{-1}
0
5
10
15
20
0
1
2
3

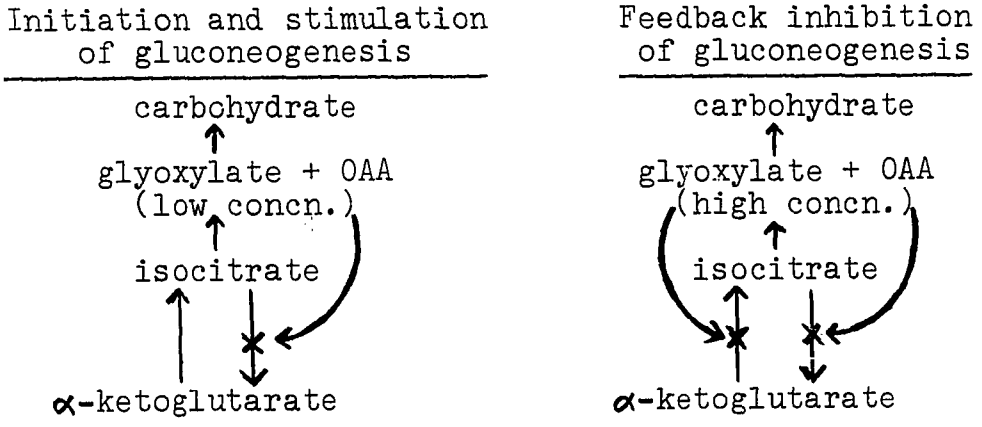
Fig. 17.--Double reciprocal plot of velocity *versus* isocitrate concentration for NADP isocitrate dehydrogenase. Assay conditions described in methods. **o**, no inhibitor; **Δ**, 0.125 mM glyoxylate plus 0.125 mM oxalacetate. Separately, 3 mM of glyoxylate or oxalacetate produced no inhibition of enzyme activity.


NADP ISOCITRATE DEHYDROGENASE
K_I = 5.5 μM
.125 mM OAA
+
.125 mM glyox.
control
1/V x 10^{-2}, UNITS^{-1}
30
20
10
0
2
4
6
8
10
12
1/[ISOCITRATE], mM^{-1}

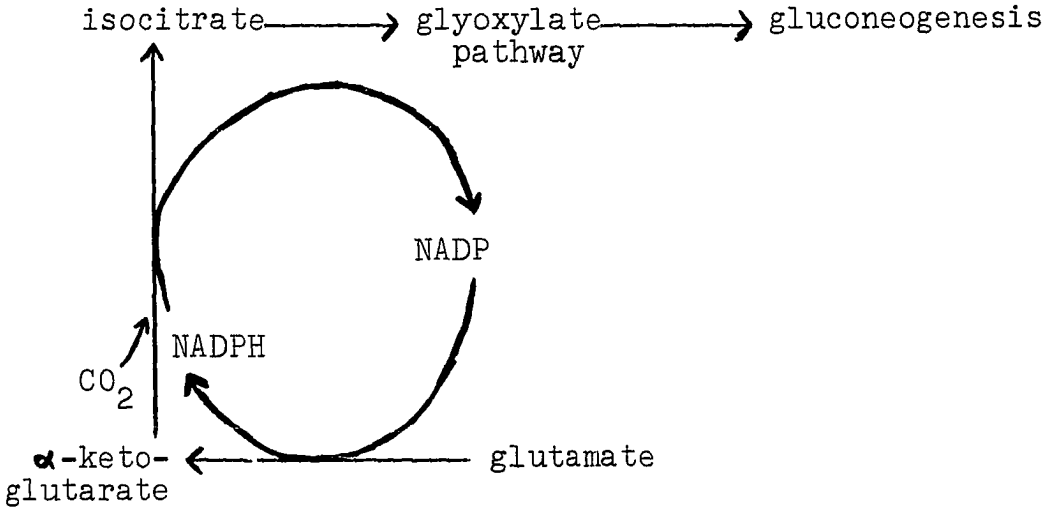
reaction in the non-gluconeogenic direction. The inhibition would prevent any "leaking back" of newly formed isocitrate into α-ketoglutarate, and thus would stimulate gluconeogenesis.

On the other hand, when glyoxylate and OAA reached a higher concentration during active gluconeogenesis, it would be advantageous to control the rate of gluconeogenesis and isocitrate formation by means of feedback inhibition. This appears to be the case in that at higher concentrations of glyoxylate and OAA, isocitrate formation is inhibited.

Control of gluconeogenesis by the regulation of NADP isocitrate dehydrogenase is made possible by the more than 200-fold difference in the ratios of K_I values for the concerted inhibitions by glyoxylate and OAA. Thus at relatively low concentrations of glyoxylate and OAA, gluconeogenesis is stimulated, whereas at relatively high concentrations of glyoxylate and OAA, gluconeogenesis is inhibited. This type of control is shown in the following diagram.



Many of the NADP isocitrate dehydrogenases isolated from other organisms have also been shown to have a correlation with increased gluconeogenesis (53). Furthermore, oxidation of glutamate to α-ketoglutarate, followed by reduction to isocitrate, would also allow the possibility of an NADP oxidation-reduction cycle as shown in the following diagram.



3. Other Effector Molecules

Phosphoenolpyruvate, a gluconeogenic precursor and end product of the glyoxylate pathway, was found to inhibit isocitrate lyase in a competitive fashion (Figure 18). Thus during gluconeogenesis accumulated PEP could be functioning as a feedback inhibitor. Further regulation of the gluconeogenic pathway was also demonstrated by the non-competitive inhibition of fructose-1,6-diphosphatase by AMP (Figure 19).

In higher animals it has been found that L-alanine can be utilized as a gluconeogenic precursor, first by being

Fig. 18.--Double reciprocal plot of velocity *versus* isocitrate concentration for isocitrate lyase. Assay conditions described in methods. ○, no inhibitor; △, 33 μM phosphoenolpyruvate.

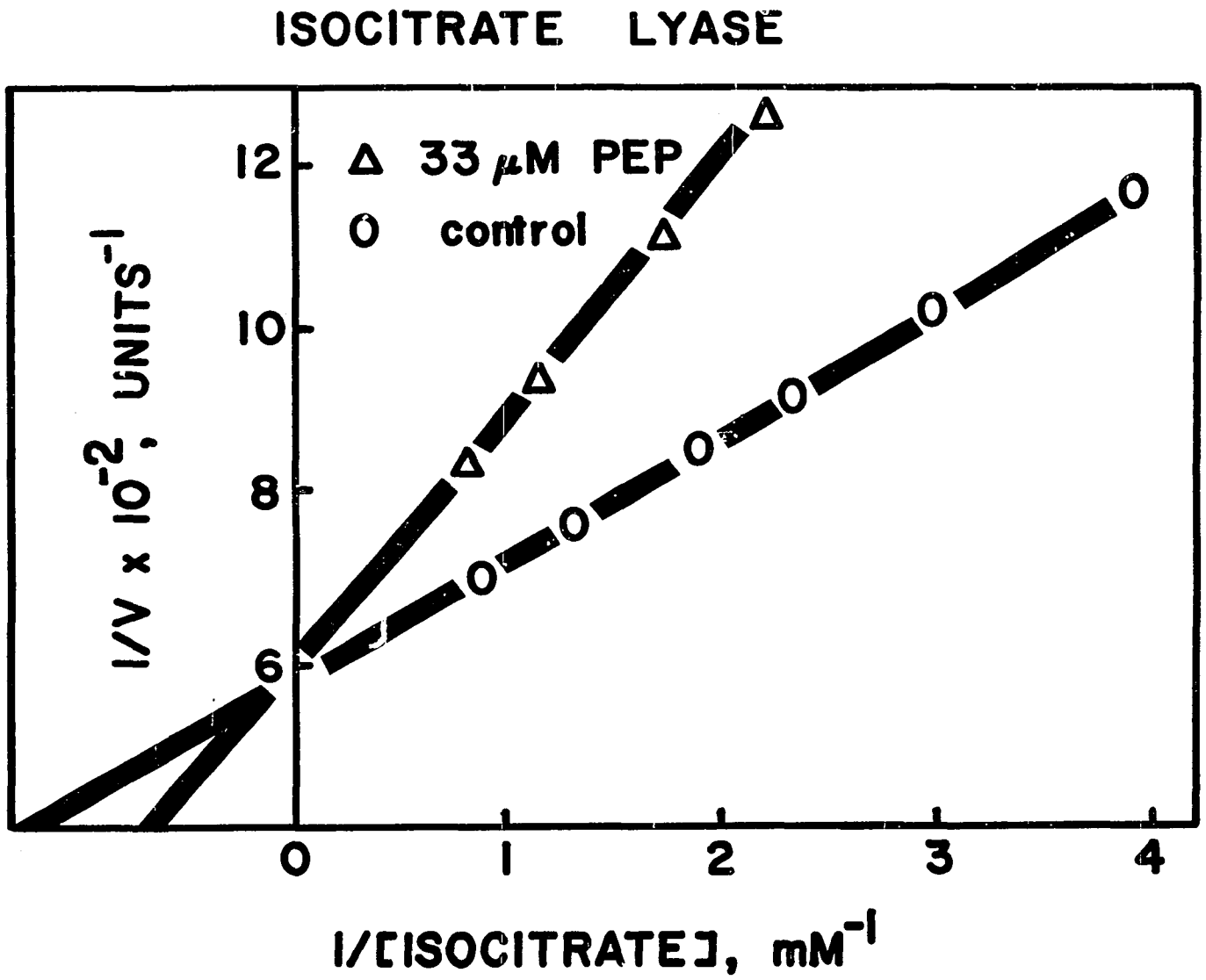
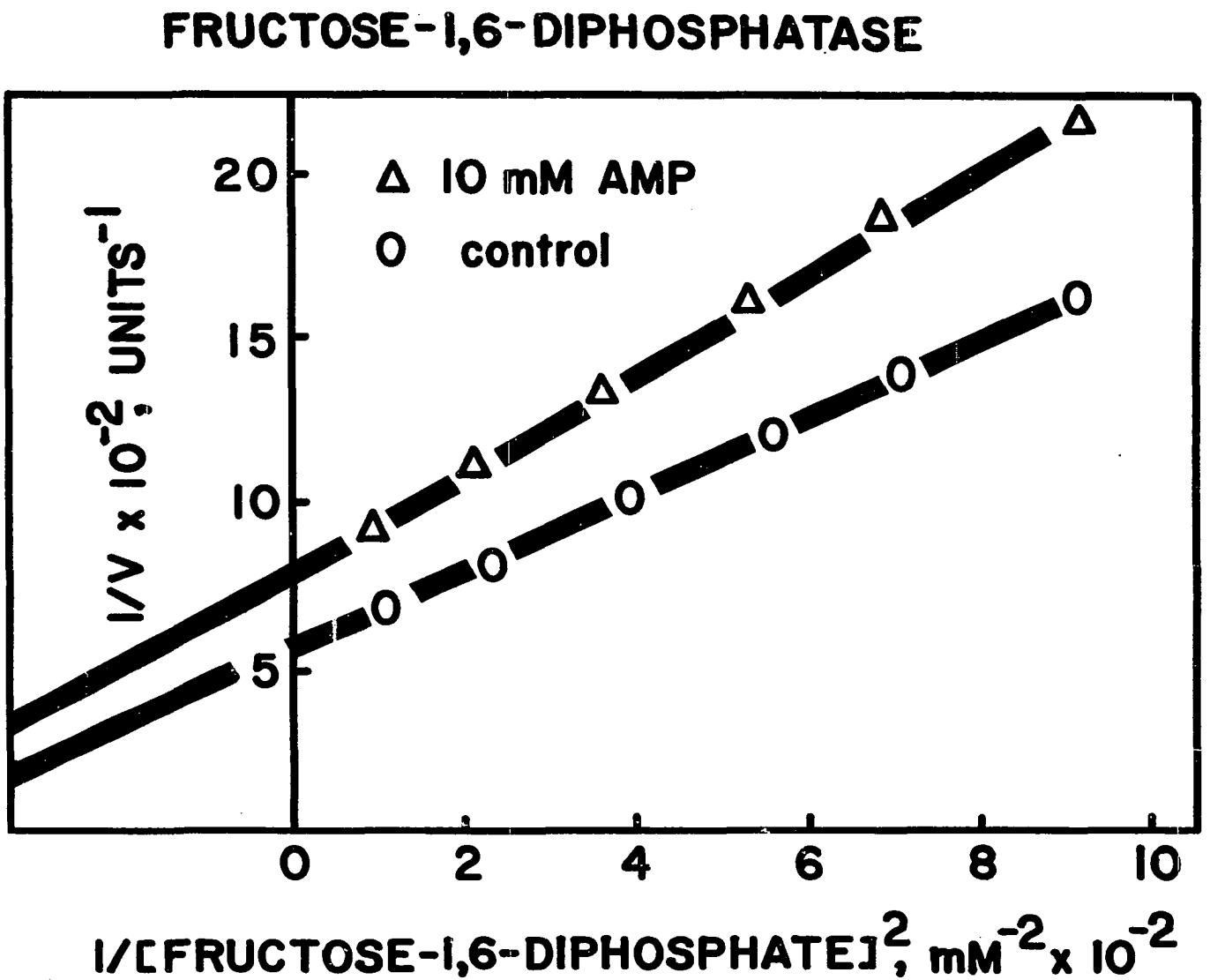

ISOCITRATE LYASE
Δ 33 μM PEP
O control
1/V x 10^-2, UNITS^-1
12
10
8
6
0
1
2
3
4
1/[ISOCITRATE], mM^-1

Fig. 19.--Double reciprocal plot of velocity *versus* fructose-1,6-diphosphate concentration for fructose-1,6-diphosphatase. Assay conditions described in methods. **o**, no inhibitor; **Δ**, 10 mM AMP.

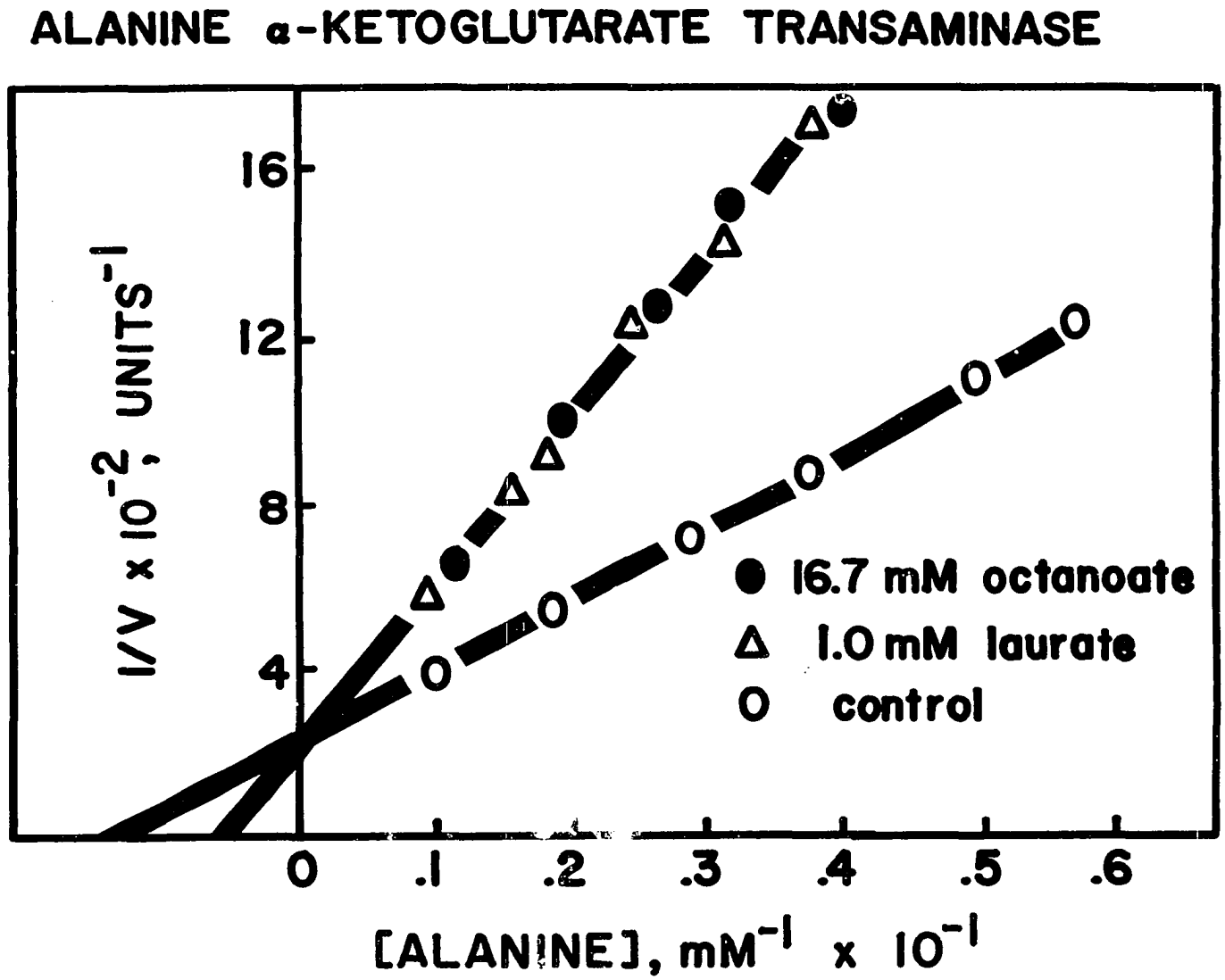

FRUCTOSE-1,6-DIPHOSPHATASE
Δ 10 mM AMP
0 control
1/V x 10⁻², UNITS⁻¹
20
15
10
5
0
2
4
6
8
10
1/[FRUCTOSE-1,6-DIPHOSPHATE]², mM⁻² x 10⁻²

transaminated to pyruvate, then carboxylated to OAA (54). Thus the question was asked whether or not L-alanine could also serve the same function in A. crystallopoietes during rod-fragmentation. From inhibitor studies it was found that FFA could inhibit alanine α-ketoglutarate transaminase in a competitive fashion (Figure 20). Since FFA are released during rod-fragmentation, and found to inhibit alanine transaminase, it is suggested that L-alanine is not a gluconeogenic precursor during rod-fragmentation. The idea is further supported by the fact that L-alanine was unable to inhibit pyruvate kinase activity of A. crystallopoietes. If the amino acid was gluconeogenic, pyruvate kinase inhibition would have been expected in order to prevent unnecessary recycling of PEP during gluconeogenesis. For example, in rat liver, where alanine is gluconeogenic, the amino acid does inhibit pyruvate kinase; whereas in rat muscle, where alanine is non-gluconeogenic, the amino acid does not inhibit pyruvate kinase (54). The pyruvate kinases of liver and muscle are probably isozymes.

4. The Phosphofructokinase of A. crystallopoietes

Glucose was found to be a poor substrate for growth of A. crystallopoietes, and enzymes such as phosphofructokinase (PFK), involved in metabolism of glucose, are believed to serve primarily a biosynthetic role, rather than a key role in the regulation of energy metabolish (30). In

Fig. 20.--Double reciprocal plot of velocity *versus* alanine concentration for alanine α-ketoglutarate transaminase. Assay conditions described in methods. ○, no inhibitor; ●, 16.7 mM sodium octanoate; △, 1.0 mM sodium laurate.


ALANINE α-KETOGLUTARATE TRANSAMINASE
1/V x 10⁻², UNITS⁻¹
16
12
8
4
0
.1
.2
.3
.4
.5
.6
[ALANINE], mM⁻¹ x 10⁻¹
16.7 mM octanoate
1.0 mM laurate
control

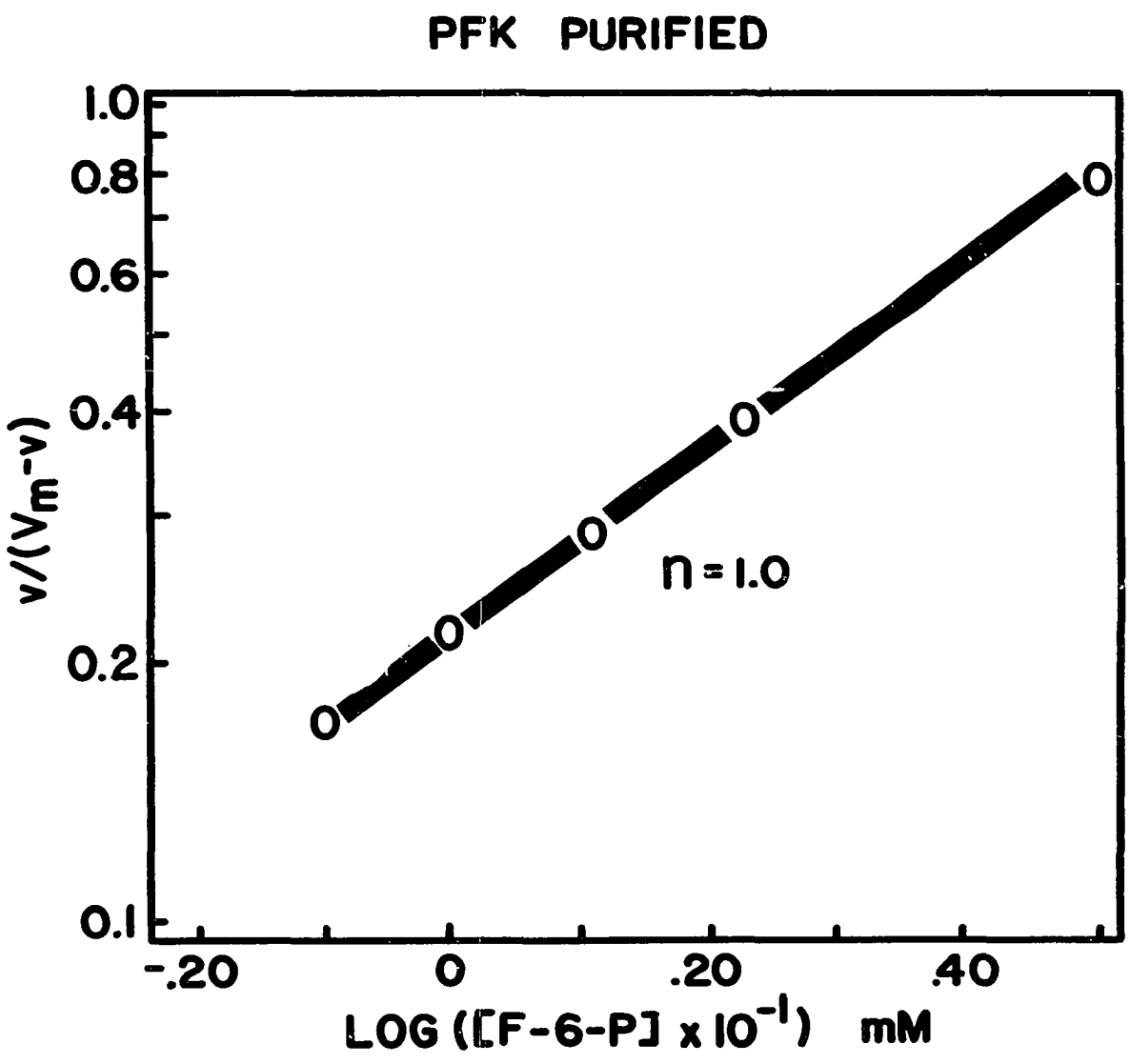
addition, succinate, amino acids, and fatty acids appear to provide the main source of energy for the morphogenesis of A. crystallopoietes (30).

Phosphofructokinase isolated from plant, animal, and bacterial sources has been found to be a highly regulated enzyme, closely associated with the control of cellular energy metabolism (50, 52). An example of such regulation is demonstrated by the response of PFK to adenylate or energy charge control (1, 3), and its response to such allosteric effectors as citrate, PEP, and NH_4^+ (27, 49, 50).

The PFK isolated from A. crystallopoietes was found to follow simple Michaelis-Menten kinetics with respect to substrate fructose-6-phosphate, and demonstrated no sigmoidal kinetics, a characteristic often associated with regulatory enzymes. Furthermore, the enzyme appeared to lack any kind of adenylate or energy charge control. Enzyme activity was not inhibited by excess ATP (10 mM), nor affected by ADP, AMP, or 3',5'-(cyclic)-AMP in concentrations up to 10 mM. In addition, enzyme activity was neither inhibited by citrate (10 mM) or PEP (10 mM), nor activated by NH_4^+ (10 mM). From a Hill plot of PFK as shown in Figure 21, an interaction coefficient of 1.0 (n=1.0) was obtained suggesting the lack of interaction between substrate binding sites, or lack of homotropic cooperativity (9).

The PFK of A. crystallopoietes was different from PFK isolated from other microbial sources in that the

Fig. 21.--Hill plot of phosphofructokinase with respect to substrate fructose-6-phosphate. A slope or interaction coefficient (n) of 1.0 was obtained. Assay conditions described in methods.


PFK PURIFIED
1.0
0.8
0.6
0.4
0.2
0.1
v/(Vm-v)
n = 1.0
-.20
0
.20
.40
LOG ([F-6-P] x 10-1) mM

enzyme followed simple Michaelis-Menten kinetics and appeared to lack an extensive type of regulation. Since glucose is not the main source of energy during the morphogenesis of A. crystallopoietes, perhaps the PFK of A. crystallopoietes has very little to do with the regulation of its own energy metabolism. This could explain the lack of response of PFK to adenylate or energy charge control, and the lack of response to other effectors such as citrate, PEP, and NH_4^+. If energy regulation occurred at sites other than PFK during morphogenesis, then one might expect a relatively simple type of PFK rather than a highly regulated PFK. The idea is further supported by the fact that Bauman and Wright (6) had recently isolated a PFK from Dictyostelium discoideum which was found to be weakly regulated, and followed simple Michaelis-Menten kinetics. A common characteristic of both A. crystallopoietes and D. discoideum is that during morphogenesis each organism utilizes fats or proteins as an internal energy source, rather than exogenous glucose. Thus the need for a highly regulated PFK to help control energy metabolism during morphogenesis of A. crystallopoietes may be totally unnecessary.

III. A Model to Explain Some of the Underlying Molecular Events of Sphere-Rod Morphogenesis of A. crystallopoietes

From the experimental data a model has been proposed to explain on a molecular level the underlying events

associated with the sphere-rod morphogenesis of A. crystallopoietes. The model is mainly concerned with two stages of growth, rod-formation and rod-fragmentation.

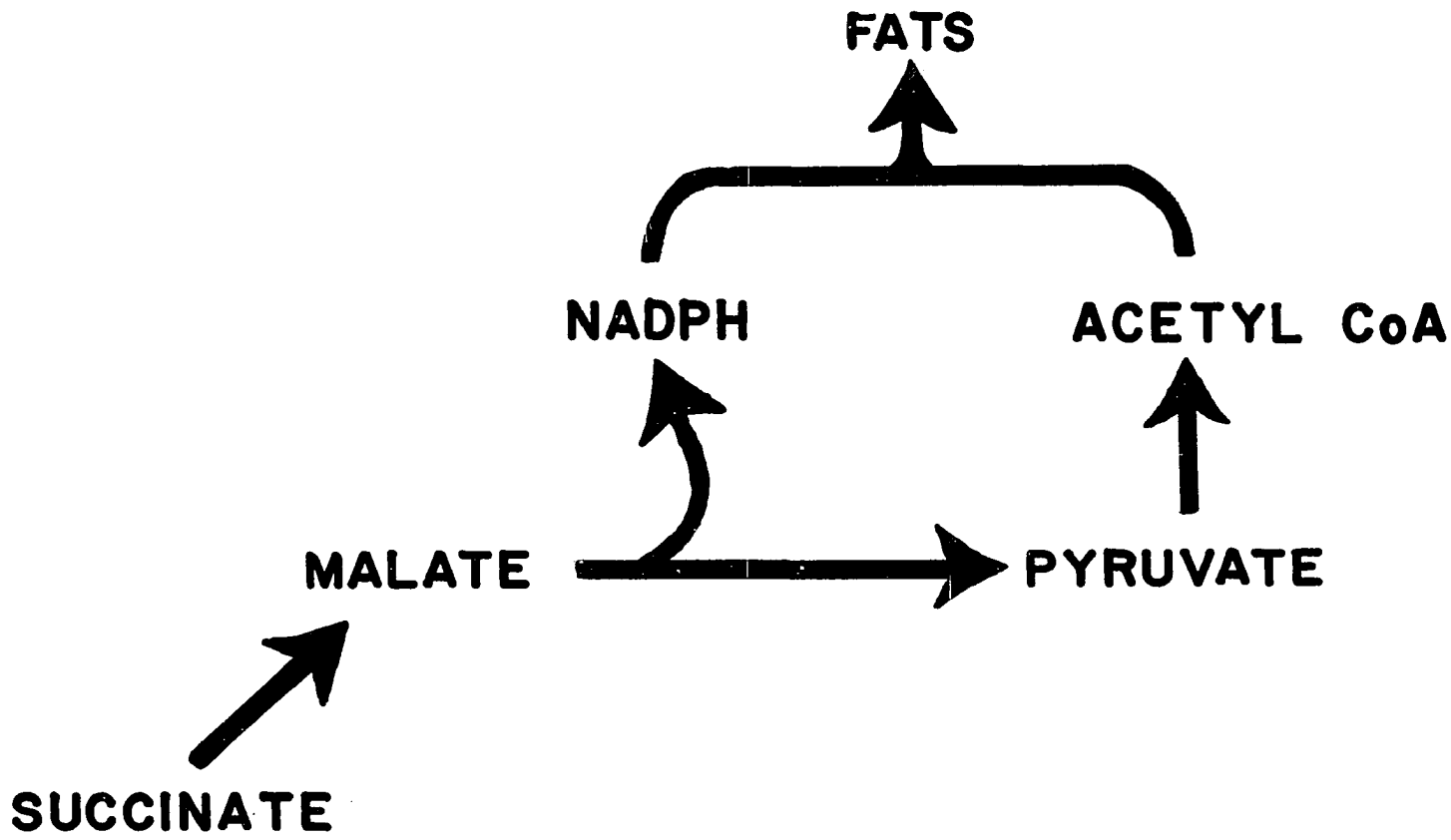
During rod-formation succinate is converted to malate, which in turn is oxidized by the malic enzyme providing the precursors for increased lipogenesis. A diagram of the chain of events is shown in Figure 22.

During rod-fragmentation accumulated fats are utilized as an internal energy source, and provide along with glutamate, necessary carbohydrate precursors for cross-septum and coccoidal wall formation. Furthermore, during rod-fragmentation active gluconeogenesis can supply the increased amounts of serine and glycine found to be present in the walls of coccoidal cells (31). A diagram of the chain of events is shown in Figure 23.

As a result of the shift in metabolism from increased lipogenesis during rod-formation, to increased gluconeogenesis during rod-fragmentation, the question was asked, what compound might act as a triggering mechanism. A likely candidate is 3',5'-(cyclic)-AMP. Cyclic AMP has already been identified as the active component which initiates the morphogenesis of D. discoideum (28), and has been shown to be the chemical messenger in the stimulation of gluconeogenesis in rat liver (60). In addition, cyclic AMP has been implicated in the activation of lipolysis of rat liver by stimulating lipase activity (56). Therefore,

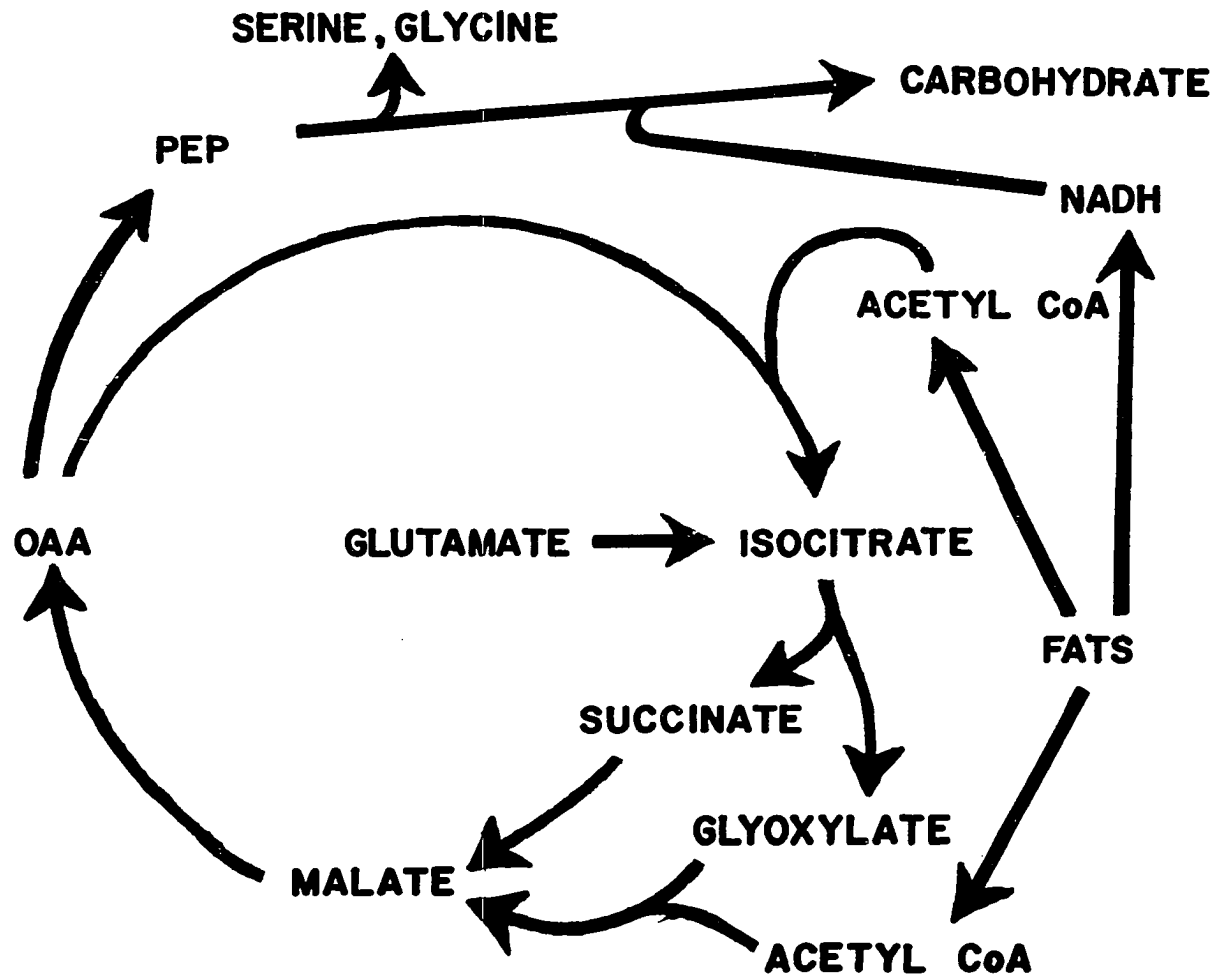
just prior to rod-fragmentation perhaps a critical concentration of cyclic AMP accumulates and is thus able to initiate rod-fragmentation.

Fig. 22.--A diagram depicting the pathways responsible for the increased rate of lipogenesis during rod-formation of Arthrobacter crystallopoietes.



ROD-FORMATION

Fig. 23.--A diagram depicting the pathways responsible for the increased rates of lipolysis and gluconeogenesis during rod-fragmentation of Arthrobacter crystallopoietes.



ROD-FRAGMENTATION

CHAPTER IV

SUMMARY

A. crystallopoietes undergoes sphere-rod morphogenesis when grown on a glucose-minimal salts medium plus succinate. Characteristic changes in the specific activity of a number of enzymes and metabolites were found as a function of the life cycle of the organism. For example, during rod-formation there was found to be an increase in the specific activity of glucose-6-phosphate dehydrogenase and the malic enzyme, while during rod-fragmentation there was an increase in specific activity of isocitrate lyase, lipase, fructose-1,6-diphosphatase, NADP isocitrate dehydrogenase, and NADP glutamate dehydrogenase.

The effect of various allosteric modifiers on enzymatic activity was also determined. It was found that FFA could selectively inhibit those enzymes involved in glycolysis or lipogenesis, but not inhibit those enzyme involved in gluconeogenesis. It was also found that glyoxylate and OAA could produce a concerted inhibition of NADP isocitrate dehydrogenase which could be of regulatory significance. In addition, it was found that glycolytic enzyme,

phosphofructokinase, followed simple Michaelis-Menten kinetics, and lacked extensive allosteric regulation. The enzyme was thus considered not to play a key role in the regulation of cellular energy metabolism.

Finally, from the experimental data a molecular model was proposed to explain some of the underlying events which are believed to occur during the sphere-rod morphogenesis of A. crystallopoietes.

BIBLIOGRAPHY

1. Atkinson, D. E. 1968. The energy charge of the adenylate as a regulatory parameter. Interaction with feedback modifiers. Biochem. 7:4030-4034.

2. Atkinson, D. E., J. A. Hathaway, and E. C. Smith. 1965. Kinetics of regulatory enzymes. Kinetic order of the yeast diphosphopyridine nucleotide isocitrate dehydrogenase reaction and a model for the reaction. J. Biol. Chem. 240:2682-2690.

3. Atkinson, D. E., and G. M. Walton. 1967. Adenosine triphosphate conservation in metabolic regulation. J. Biol. Chem. 242:3239-3241.

4. Baldwin, R. L., and L. P. Milligan. 1966. Enzymatic changes associated with the initiation and maintenance of lactation in the rat. J. Biol. Chem. 241:2058-2066.

5. Ball, E. G. 1966. Regulation of fatty acid synthesis in adipose tissue, p. 3-18. In G. Weber ed., Advances in Enzyme Regulation, vol. 4. Pergamon Press, Oxford.

6. Bauman, P., and B. E. Wright. 1968. The phosphofructokinase of Dictyostelium discoideum. Biochem. 7:3653-3651.

7. Benziman, M. 1966. Implication of direct phosphorylation of pyruvate in cellulose synthesis by Acetobacter xylinum. Biochem. Biophys. Res. Commun. 24:391-394.

8. Berman, K., N. Itada, and M. Cohn. 1967. On the mechanism of ATP cleavage in phosphoenolpyruvate synthase reaction of Escherichia coli. Biochim. Biophys. Acta. 141:214-216.

9. Blangy, D., H. Buc, and J. Monod. 1968. Kinetics of allosteric interactions of phosphofructokinase from Escherichia coli. J. Mol. Biol. 31:13-35.

10. Bridgeland, E. S., and K. M. Jones. 1967. Formation of dicarboxylic acids and phosphoenolpyruvate in Arthrobacter globiformis. Biochem. J. 104:9P-10P.

11. Buchanan, B. B., and M. C. W. Evans. 1966. The synthesis of phosphoenolpyruvate from pyruvate and ATP by extracts of photosynthetic bacteria. Biochem. Biophys. Res. Commun. 22:484-487.

12. Chang, H. C., and M. D. Lane. 1966. The enzymatic carboxylation of phosphoenolpyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. J. Biol. Chem. 241:2413-2420.

13. Clark, J. B., and C. Aldridge. 1960. Fat bodies in Nocardia corallina. J. Bacteriol. 79:756-757.

14. Conn, H. J., J. W. Bartholomew, and M. W. Jennison. 1957. Stain for fat droplets, p. 30. In H. J. Conn ed., Manual of Microbiological Methods. McGraw-Hill Book Co., Inc., New York.

15. Conn, E. E., and P. K. Stumpf. 1966. Lipid metabolism, p. 274-294. In, Outlines of Biochemistry. John Wiley & Sons, Inc., New York.

16. Davis, J. E., and D. M. Gibson. 1969. Regulation of the metabolism of rabbit liver mitochondria by long chain fatty acids and other uncouplers of oxidative phosphorylation. J. Biol. Chem. 244:161-170.

17. Dixon, G. H., and H. L. Kornberg. 1964. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. 72:3P.

18. Dixon, M., and E. C. Webb. 1964. Enzyme inhibitors, p. 315-359. In, Enzymes. Academic Press Inc., New York.

19. Ensign, J. C., and R. S. Wolfe. 1964. Nutritional control of morphogenesis in Arthrobacter crystallopoietes. J. Bacteriol. 87:924-932.

20. Friedman, B., E. H. Goodman, and S. Weinhouse. 1967. Effects of insulin and fatty acid on gluconeogenesis in the rat. J. Biol. Chem. 242:3620-3627.

21. Goodridge, A. G. 1968. Citrate-cleavage enzyme, 'malic' enzyme and certain dehydrogenases in embryonic and growing chicks. Biochem. J. 108:663-666.

22. Hanson, R. S., and D. P. Cox. 1967. Effect of different nutritional conditions on the synthesis of tricarboxylic acid cycle enzymes. J. Bacteriol. 93: 1777-1787.

23. Harada, K., and R. G. Wolfe. 1968. Malic dehydrogenase. VI. A study of hydroxymalonate inhibition. J. Biol. Chem. 243-4123-4130.

24. Hittle, L. L. 1963. Changes in the free amino acid pool in Nocardia corallina. Thesis submitted for Master of Science Degree, University of Oklahoma.

25. Hogg, J. F., and H. L. Kornberg. 1963. The metabolism of C_2-compounds in micro-organisms. IX. Role of the glyoxylate cycle in protozoal glyconeogenesis. Biochem. J. 86:462-468.

26. Hsu, Y., H. A. Lardy, and W. W. Cleland. 1967. Pigeon liver malic enzyme. J. Biol. Chem. 242:5315-5322.

27. Kelly, G. J., and J. P. Turner. 1968. Inhibition of pea-seed phosphofructokinase by phosphoenolpyruvate. Biochem. Biophys. Res. Commun. 30:195-199.

28. Konijn, T. M., J. G. C. Van De Meene, J. T. Bonner, and D. S. Barkley. 1967. The acrasin activity of adenosine-3',5'-cyclic phosphate. Proc. Natl. Acad. Sci. 58:1152-1154.

29. Kornberg, H. L. 1966. The role and control of the glyoxylate cycle in Escherichia coli. Biochem. J. 99:1-11.

30. Krulwich, T. A., and J. C. Ensign. 1969. Alteration of the glucose metabolism of Arthrobacter crystallopoietes by compounds which induce sphere-rod morphogenesis. J. Bacteriol. 97:526-534.

31. Krulwich, T. A., J. C. Ensign, D. J. Tipper, and J. L. Strominger. 1967. Sphere-rod morphogenesis in Arthrobacter crystallopoietes. I. Cell wall composition and polysaccharides of the peptidoglycan. J. Bacteriol. 94:734-740.

32. Kuby, S. A., and E. A. Noltman. 1966. Glucose-6-phosphate dehydrogenase (crystalline) from Brewer's yeast, p. 116-125. In W. A. Wood ed., Methods in Enzymology, vol. IX. Academic Press, New York.

33. Ling, M. A., V. Paetkau, F. Marcus, and H. A. Lardy. 1966. Phosphofructokinase. I. Skeletal muscle, p. 425-429. In W. A. Wood ed., Methods in Enzymology, vol. IX. Academic Press, New York.

34. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-275.

35. Ochoa, S. 1955. "Malic" enzyme from pigeon liver and wheat germ, p. 739-748. In S. P. Colowick and N. O. Kaplan ed., Methods in Enzymology, vol. I. Academic Press Inc., New York.

36. Okuda, H., and S. Fujii. 1968. Relationship between lipase and esterase. J. Biochem. 64:377-392.

37. Pande, S. V., R. P. Kahn, and T. A. Venkitasubramanian. 1964. Nicotinamide adenine dinucleotide phosphate-specific dehydrogenases in relation to lipogenesis. Biochim. Biophys. Acta. 84:239-250.

38. Plaut, G. W. E., and S. C. Sung. 1955. Diphosphopyridine nucleotide isocitric dehydrogenase from animal tissues, p. 710-714. In S. P. Colowick and N. O. Kaplan ed., vol. I. Academic Press Inc., New York.

39. Reeves, W. J., and G. M. Fimognari. 1966. L-lactic dehydrogenase:heart (H_4), p. 288-294. In W. A. Wood ed., Methods in Enzymology, vol. IX. Academic Press, New York.

40. Rose, F. L., and H. L. Lewis. 1968. Changes in weight and free fatty acid concentration of fat bodies of paedogenic Ambystoma tigrinum during vitellogenesis. Compar. Biochem. Biophysiol. 26:149-154.

41. Rosen, O. M., S. M. Rosen, and B. L. Horecker. 1966. Fructose-1,6-diphosphatase. II. Candida utilis, p. 632-636. In W. A. Wood ed., Methods in Enzymology, vol. IX. Academic Press, New York.

42. Rowsell, E. V. 1962. Transaminases in animal tissue homogenates, p. 685-697. In S. P. Colowick and N. O. Kaplan ed., Methods in Enzymology, vol. V. Academic Press, New York.

43. Scrutton, M. C., and M. F. Utter. 1968. The regulation of glycolysis and gluconeogenesis in animal tissues, p. 249-302. In P. D. Boyer ed., Annual Review of Biochemistry, vol. 37. Annual Reviews Inc., Palo Alto, California.

44. Slein, M. W. 1955. Phosphohexoisomerase from muscle, p. 299-306. In S. P. Colowick and N. O. Kaplan ed., Methods in Enzymology, vol. I. Academic Press Inc., New York.

45. Strecker, H. J. 1955. L-glutamic dehydrogenase from liver, p. 220-225. In S. P. Colowick and N. O. Kaplan ed., Methods in Enzymology, vol. I. Academic Press Inc., New York.

46. Struck, E., J. Ashmore, and O. Wieland. 1966. Effects of glucagon and long chain fatty acids on glucose production by isolated perfused rat liver, p. 219-224. In G. Weber ed., Advances in Enzyme Regulation, vol. 4. Pergamon Press, Oxford.

47. Taylor, G. B., E. Bailey, and W. Bartley. 1967. Changes in hepatic lipogenesis during developement of the rat. Biochem. J. 105:717-722.

48. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. In, Manometric Techniques. Burgess Publishing Co., Minneapolis, Minnesota.

49. Underwood, A. H., and E. A. Newsholme. 1967. Some properties of phosphofructokinase from kidney cortex and their relation to glucose metabolism. Biochem. J. 104:206-299.

50. Uyeda, K., and E. Racker. 1965. Regulatory mechanisms in carbohydrate metabolism. VII. Hexokinase and phosphofructokinase. J. Biol. Chem. 240:4682-4688.

51. Valentine, W. N., and K. R. Tanaka. 1966. Pyruvate kinase: clinical aspects, p. 468-473. In W. A. Wood ed., Methods in Enzymology, vol. IX. Academic Press, New York.

52. Walker, P. R., and E. Bailey. 1969. A comparison of the properties of phosphofructokinase of the fat body and flight muscle of the adult male desert lucust. Biochem J. 111:365-369.

53. Walter, P., V. Paetkan, and H. A. Lardy. 1966. Paths of carbon in gluconeogenesis and lipogenesis. III. The role and regulation of mitochondrial processes involved in supplying precursors of phosphoenol-pyruvate. J. Biol. Chem. 241:2523-2532.

54. Weber, G., M. A. Lea, and N. B. Stamm. 1968. Sequential feedback inhibition and regulation of liver carbohydrate metabolism through control of enzyme activity, p. 101-123. In G. Weber ed., Advances in Enzyme Regulation, vol. 6. Pergamon Press, Oxford.

55. Weber, G., M. A. Lea, H. J. H. Convery, and N. B. Stamm. 1967. Regulation of gluconeogenesis and glycolysis: studies of the mechanism controlling enzyme activity, p. 257-299. In G. Weber ed., Advances in Enzyme Regulation, vol. 5. Pergamon Press, Oxford.

56. Wing, D. R., and D. S. Robinson. 1968. Clearing-factor lipase in adipose tissue. A possible role of adenosine 3', 5'-(cyclic)-monophosphate in the regulation of its activity. Biochem. J. 109:841-849.

57. Wise, E. M., and E. G. Ball. 1964. Malic enzyme and lipogenesis. Proc. Natl. Acad. Sci. 52:1255-1263.

58. Wright, B. E. 1964. Biochemistry of morphogenesis, p. 1-17. In M. Florkin and H. S. Mason ed., Comparative Biochemistry, vol. VI. Academic Press, New York.

59. Wright, B. E. 1966. Multiple causes and controls in differentiation. Science. 153:830-837.

60. Yeung, D., and I. T. Oliver. 1968. Induction of phosphopyruvate carboxylase in neonatal rat liver by adenosine 3', 5'-cyclic monophosphate. Biochem. 7:3231-3239.

61. Young, J. W., E. Shargo, and H. A. Lardy. 1964. Metabolic control of enzymes involved in lipogenesis and gluconeogenesis. Biochem. 3:1687-1692.